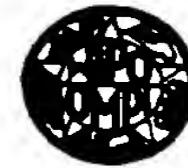


PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification 6 : C12N 15/24, C07K 14/54, A61K 38/20, C12N 15/62, G01N 33/68</p>		A1	<p>(11) International Publication Number: WO 97/41232 (43) International Publication Date: 6 November 1997 (06.11.97)</p>
<p>(21) International Application Number: PCT/US97/06931 (22) International Filing Date: 25 April 1997 (25.04.97)</p>		<p>(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p>	
<p>(30) Priority Data: 60/016,634 26 April 1996 (26.04.96) US</p>		<p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(71) Applicant: BETH ISRAEL DEACONESS MEDICAL CENTER (US/US); 330 Brookline Avenue, Boston, MA 02215 (US).</p>			
<p>(72) Inventors: STROM, Terry; 22 Kennard Road, Brookline, MA 02146 (US). MASLINSKI, Wlodzimerz; 22 Kirkwood Road, Brighton, MA 02135 (US).</p>			
<p>(74) Agent: FREEMAN, John, W.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).</p>			
<p>(54) Title: ANTAGONISTS OF INTERLEUKIN-15</p>			
<p>(57) Abstract</p>			
<p>Disclosed herein are mutant IL-15 polypeptides and methods for using these polypeptides to modulate the immune response in a patient.</p>			

- 2 -

Summary of the Invention

Mutants of the cytokine IL-15 that bind the IL-15 receptor complex with an affinity similar to wild-type IL-15, but fail to activate signal transduction, have 5 been produced. These mutant polypeptides compete effectively with wild-type IL-15 and can block one or more of the events that normally occur in response to IL-15 signalling, such as cellular proliferation. By modulating the events mediated by the IL-15 receptor 10 complex, mutant IL-15 polypeptides can modulate the immune response, and thus are therapeutically useful.

Mutant IL-15 polypeptides can have several characteristics that are advantageous in the context of treatment regimes. First, they are less likely to be 15 immunogenic because they can differ from wild-type IL-15 by only a few substituted residues. Second, IL-15 mutants bind IL-15R α with the same high affinity as wild type IL-15 and thus, can compete effectively for the receptor. Third, IL-15 mutants can be easily modified to 20 remain active in the circulation for a prolonged period of time, or to produce a lytic response in cells to which they bind. In addition, the IL-15 receptor alpha (IL-15R α) polypeptide is expressed upon activated or malignant immune cells, but not on resting immune cells. 25 Thus, the mutant polypeptide of the invention can be used to specifically target activated or malignant immune cells.

Other features and advantages of the invention will be apparent from the following detailed description, 30 and from the claims. Although materials and methods similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred materials and methods are described below.

- 1 -

ANTAGONISTS OF INTERLEUKIN-15

Related Application(s)

5 This application claims benefit from a provisional application that was filed on April 26, 1996, and assigned U.S. Serial No. 60/016,634.

Field of the Invention

10 The field of the invention is cytokine-mediated therapeutics.

Background of the Invention

15 An effective immune response begins when an antigen or mitogen triggers the activation of T cells. In the process of T cell activation, numerous cellular changes occur, which include the expression of cytokines and cytokine receptors. One of the cytokines involved in the immune response is interleukin-15 (IL-15). IL-15 is a T cell growth factor that stimulates the proliferation and differentiation of B cells, T cells, natural killer 20 (NK) cells, and lymphocyte-activated killer (LAK) cells to *in vitro*. *In vivo*, the proliferation of these cell types enhances the immune response.

25 IL-15 binds to a heterotrimeric receptor that consists of the IL-2R β subunit, the IL-2R γ subunit, and a unique IL-15R α subunit.

Patients benefit from suppression of the immune response in a number of circumstances, for example, in the event of organ transplantation or autoimmune disease.

30 In other circumstances, for example when select immune cells have become malignant or autoaggressive, it is beneficial to actively destroy them.

- 4 -

incorporated radioactivity was determined by scintillation counting.

Figs. 4A-4C are a series of plots depicting the expression of IL-15R α subunits on human PBMCs by flow cytometry analysis of stained cells. Freshly isolated or PHA pre-activated PBMCs were washed and incubated with medium alone (thin line) or FLAG-HMK-IL-15 (thick line) followed by anti-FLAG biotinylated Ab and Streptavidin-RED670 (Fig. 4A). The data presented in Fig. 4B were obtained from washed PBMCs that were preincubated with media alone (left-hand side) or media containing 2 μ g of human recombinant IL-15 (right-hand side) for 20 minutes at 4°C before the addition of FLAG-HMK-IL-15. For simplicity, graphs represent specific binding of FLAG-15-HMK-IL-15; non-specific binding was subtracted. The data presented in Fig. 4C were obtained from PBMCs that were preincubated with phycoerythrin conjugated anti-CD4 or anti-CD8 for 30 minutes before the addition of FLAG-HMK-IL-15.

Figs. 5A-5C are a series of plots generated by fluorescence activated cell sorting (FACS) analysis of PHA-activated PBMCs stained with FLAG-HMK-IL-15 proteins and anti-CD254 (IL-2R α chain) monoclonal antibodies. Fig. 5A demonstrates the existence of IL-2R α $^{+}$ cells that do not bind FLAG-HMK-IL-15. Fig. 5B demonstrates that almost all PBMCs stimulated with PHA for only one day express either IL-15R α or IL-2R β subunits, but not both. Fig. 5C demonstrates that a larger population of IL-15R α $^{+}$ and IL-2R β $^{+}$ cells (double positive) are present 3 days following PHA stimulation.

Figs. 6A-6D are a series of plots generated by fluorescence activated cell sorting (FACS). To determine the effect of immunosuppressant drugs on the mitogen-induced expression of IL-15R α , PBMCs were preincubated with cyclosporin (CsA; Fig. 6B), rapamycin (RAPA; Fig.

- 3 -

Brief Description of the Drawings

Fig. 1 is a photograph of a Western blot depicting the bacterially-produced, affinity-purified FLAG-HMK-IL-15 fusion protein. Proteins were separated by 5 electrophoresis through a 20% SDS-polyacrylamide gel, visualized by staining with Coomassie blue (lane a) and transferred to PVDF membranes. The membranes were exposed to antibodies against the FLAG peptide (lane b) and human IL-15 (lane c). In lane (d), eluates from an 10 anti-FLAG affinity column were incubated with heart muscle kinase and [³²P]- γ -ATP for 30 minutes before electrophoresis.

Fig. 2 is an autoradiograph depicting proteins that were extracted from PHA-stimulated PBMCs that were 15 incubated with [³²P]-FLAG-HMK-IL-15, cross-linked with DSS, and separated by SDS-PAGE under reducing conditions. A 75-80 kDa band is apparent (lane 1). To control for nonspecific cross-linking, replicates were incubated with [³²P]-FLAG-HMK-IL-15 in the presence of a molar excess of 20 IL-15 (lane 2).

Fig. 3A is a bar graph depicting the mitogenic response of PHA-stimulated PBMCs that were stimulated with either buffer alone ("none"), FLAG peptide (10⁻⁹ M), IL-2 (10⁻⁹ M) or FLAG-HMK-IL-15 (10⁻⁹ M). The cultured 25 cells were "pulsed" with [³H]-TdR, and the amount of incorporated radioactivity was determined by scintillation counting.

Fig. 3B is a bar graph depicting the mitogenic response of BAF-BO35 cells transfected with pRcCMV 30 (control; left-hand panel) or with pRcCMV-IL-2R β (encoding the wild-type human IL-2R β subunit; right-hand panel) and incubated with medium alone ("none"), media containing IL-2R β IL-2 (50 U/ml), or FLAG-HMK-IL-15 (10 ng/ml), and pulsed with [³H]-TdR (b). The cultured cells 35 were "pulsed" with [³H]-TdR, and the amount of

- 6 -

exogenously added cytokines. Error bars indicate the mean \pm standard deviation.

Fig. 11 is a graph depicting the effect of cyclosporin-A on the proliferation of PHA-stimulated 5 PBMCS. The cells were incubated with increasing concentrations of cyclosporine-A for 15 minutes at 37°C, followed by stimulation with IL-2 or IL-15 for 38 hours, and a standard proliferation assay was performed. In the control experiment, cells were cultured in media lacking 10 exogenously added cytokines. Error bars indicate the mean \pm standard deviation.

Fig. 12 is a pair of graphs depicting the proliferative response of BAF-BO3 cells that express the wild-type IL-2R β subunit (WT, Δ --- Δ), the mutant IL-2R β 15 subunit that lacks serine-rich region (S, \blacksquare --- \blacksquare), or a control vector (V, \bullet --- \bullet). The cells were incubated with IL-2 (upper graph) or IL-15 (lower graph) for 38 hours and a standard proliferation assay was performed. Error bars indicate the mean \pm standard deviation.

20 Fig. 13 is a representation of the wild-type IL-15 nucleic acid and predicted amino acid sequence.

Fig. 14 is a representation of the mutant IL-15 nucleic acid and predicted amino acid sequence. The wild-type codon encoding glutamine at position 149, CAG, 25 and the wild-type codon encoding glutamine at position 156, CAA, have both been changed to GAC, which encodes aspartate. (These positions (149 and 156) correspond to positions 101 and 108, respectively, in the mature IL-15 polypeptide, which lacks a 48-amino acid signal 30 sequence).

Fig. 15 is a bar graph depicting the proliferative response of BAF-BO3 cells cultured in the presence of IL-15 related polypeptides. Abbreviations: WT=BAF-BO3 cells transfected with pRcCMV-IL-2R β (encoding the human 35 IL-2R β subunit); V=BAF-BO3 cells transfected with pRcCMV-

- 5 -

6C), or dexamethasone (Dex; Fig. 6D) for 20 minutes before the addition of PHA and then cultured for 3 days. The control, stimulation with PHA only, is shown in Fig. 6A. The level of IL-15R α expression was detected using 5 FLAG-HMK-IL-15, anti-FLAG biotinylated Ab, streptavidin-RED670, and FACS analysis.

Fig. 7 is a bar graph depicting the proliferative response of human PBMCs that were pre-treated with immunosuppressive drugs and PHA and then treated with 10 PHA, IL-2 or IL-15. Cells were harvested after a 4 hour pulse of [3 H]-TdR and cell-incorporated radioactivity was measured in a scintillation counter.

Fig. 8A is a diagram of the protocol used to induce IL-2 and IL-15 responsive PBMCs.

15 Fig. 8B is a graph depicting the proliferative response of PBMCs that were pre-stimulated with PHA for 3 days and then cultured in the presence of IL-2 (Δ ; \square) or IL-15 (\blacktriangle ; \blacksquare) for 2 days, and then tested for secondary response to IL-2 or IL-15 according to the protocol 20 illustrated in Fig. 8A. Arrow indicates that the cytokine was present during secondary stimulation. Error bars indicate the mean \pm standard deviation.

Fig. 9 is a line graph. PHA-stimulated PBMCs were incubated with genistein at various doses for 15 minutes 25 at 37°C followed by stimulation with IL-2 or IL-15 for 38 hours in a standard proliferation assay. Control cells were not treated with genistein. Error bars indicate the mean \pm standard deviation.

Fig. 10 is a graph depicting the effect of 30 rapamycin on the proliferation of PHA-stimulated PBMCs. Cells were incubated with increasing concentrations of rapamycin for 15 minutes at 37°C, followed by stimulation with IL-2 (\circ - \circ) or IL-15 (\square - \square) for 38 hours, and a standard proliferation assay was performed. In the 35 control experiment, cells were cultured in media lacking

- 8 -

(tracing alone) or with medium containing the FLAG-HMK-IL-15 polypeptide (tracing filled in). The cells were then incubated with the biotinylated anti-FLAG antibody and stained with RED670-conjugated streptavidin. The 5 stained cells were analyzed by flow cytometry.

Detailed Description

Mutant IL-15 polypeptides can suppress IL-15 dependent immune responses by selectively inhibiting the activity of cells that bind wild-type IL-15. The mutant 10 can also be used to kill the cells to which it binds.

Accordingly, the invention features a substantially pure polypeptide that is a mutant of wild-type IL-15, nucleic acid molecules that encode this polypeptide, and cells that express the mutant 15 polypeptide. Methods of treatment, wherein a mutant IL-15 polypeptide is administered to suppress the immune response (for example, in the event of an autoimmune disease) or to kill IL-15-binding cells (for example, in the event of unwanted cellular proliferation of 20 IL-15-binding cells) are also within the scope of the invention. ~~ISSUE DATE - APR 2004 - 10:12~~
~~10:12 AM 04 APR 2004~~

Mutant IL-15 Polypeptides

The invention features a substantially pure polypeptide that is a mutant of wild-type IL-15, and 25 which can function as an antagonist of wild-type IL-15. The "wild-type IL-15 polypeptide" referred to herein is a polypeptide that is identical to a naturally-occurring IL-15 (interleukin-15) polypeptide. IL-15 has been characterized as a T cell growth factor that stimulates 30 the proliferation and differentiation of B cells, T cells, natural killers (NK cells), and lymphocyte-activated killers (LAK) cells in vitro. In contrast, a "mutant IL-15 polypeptide" is a polypeptide that has at

- 7 -

0 (plasmid without insert); none=incubation of cells in medium without added interleukin; DM=incubation of cells in medium containing the IL-15 double mutant (FLAG-HMK-IL-15-Q149D-Q156D); 149=incubation of cells in medium 5 containing the IL-15 single mutant (FLAG-HMK-IL-15-Q149D).

Fig. 16 is a bar graph depicting the proliferative response of PHA-stimulated human PBMCs cultured in the presence of IL-15 related proteins. Abbreviations:
10 WT=BAF-BO3 cells transfected with pRcCMV-IL-2R β (encoding the human IL-2R β subunit); V=BAF-BO3 cells transfected with pRcCMV-0 (plasmid without insert); none=incubation of cells in medium without added interleukin; DM=incubation of cells in medium containing the IL-15 double mutant (FLAG-HMK-IL-15-Q149D-Q156D); 149=incubation of cells in medium containing the IL-15 single mutant (FLAG-HMK-IL-15-Q149D); 156=incubation of cells in medium containing the IL-15 single mutant (FLAG-HMK-IL-15-Q156D).

20 Fig. 17 is a series of plots demonstrating the ability of the FLAG-HMK-IL-15 double mutant to bind PHA-activated human PBMCs. PHA-activated PBMCs were washed and incubated with medium alone (a) or with FLAG-HMK-IL-15 double mutants (b), followed by anti-FLAG 25 biotinylated Ab and streptavidin-RED670. The stained cells were analyzed by flow cytometry. In (c), (a) and (b) are overlapped (control=thin line; FLAG-HMK-IL-15- double mutant=thick line).

Fig. 18 is a series of plots demonstrating the 30 ability of the wild-type FLAG-HMK-IL-15 polypeptide to bind leukemia cells. The cells treated were from the leukemic cell lines MOLT-14, YT, HUT-102, and from cell lines currently being established at Beth Israel Hospital (Boston, MA), and designated 2A and 2B. The cultured 35 cells were washed and incubated with either medium alone

- 10 -

reference polypeptide over its entire length. Of course, many other polypeptides will meet the same criteria.

Sequence identity is typically measured using sequence analysis software such as the Sequence Analysis Software Package of the Genetics Computer Group at the University of Wisconsin Biotechnology Center (1710 University Avenue, Madison, WI 53705), with the default parameters thereof.

The mutant polypeptide can be at least 65%, 10. preferably at least 80%, more preferably at least 90%, and most preferably at least 95% (e.g., 99%) identical to wild-type IL-15. The mutation can consist of a change in the number or content of amino acid residues. For example, the mutant IL-15 can have a greater or a lesser 15. number of amino acid residues than wild-type IL-15.

Alternatively, or in addition, the mutant polypeptide can contain a substitution of one or more amino acid residues that are present in the wild-type IL-15. The mutant IL-15 polypeptide can differ from wild-type IL-15 by the 20 addition, deletion, or substitution of a single amino acid residue; for example, a substitution of the residue at position 156. Similarly, the mutant polypeptide can differ from wild-type by a substitution of two amino acid residues; for example, the residues at positions 156 and 25 149. For example, the mutant IL-15 polypeptide can differ from wild-type IL-15 by the substitution of aspartate for glutamine at residues 156 and 149 (as shown in Figures 14 and 15). Additions, deletions, and substitutions of more than two residues, and 30 substitutions at other positions are likely to produce a similarly useful (i.e., therapeutically effective) mutant IL-15 polypeptide.

The substituted amino acid residue(s) can be, but are not necessarily, conservative substitutions, which 35 typically include substitutions within the following

- 9 -

least one mutation relative to wild-type IL-15 and that inhibits any *in vivo* or *in vitro* activity that is characteristic of wild-type IL-15.

A mutant IL-15 polypeptide that can be used 5 according to the present invention will generally blocks at least 40%, more preferably at least 70%, and most preferably at least 90% of one or more of the activities of the wild-type IL-15 molecule. The ability of a mutant IL-15 polypeptide to block wild-type IL-15 activity can 10 be assessed by numerous assays, including the BAF-B03 cell proliferation assay described herein (in which the cells were transfected with a construct encoding IL-2R β).

The mutant polypeptides of the invention can be referred to as exhibiting a particular percent identity 15 to wild-type IL-15. When examining the percent identity between two polypeptides, the length of the sequences compared will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 20 amino acids. It is to be understood that

The term "identity" as used herein in reference to polypeptide or DNA sequences, refers to the subunit sequence identity between two molecules. When a subunit position in both of the molecules is occupied by the same 25 monomeric subunit (i.e., the same amino acid residue or nucleotide), then the molecules are identical at that position. The similarity between two amino acid or two nucleotide sequences is a direct function of the number of identical positions. Thus, a polypeptide that is 50% 30 identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely identical to a 50 amino acid long portion of the reference polypeptide. It might also be a 100 amino acid long polypeptide that is 50% identical to the

- 12 -

polypeptide; as described further below, native activity is not necessary or desired in all cases.

The Fc region can be "lytic" or "non-lytic." A non-lytic Fc region typically lacks a high affinity Fc receptor binding site and a C'1q binding site. The high affinity Fc receptor binding site of murine IgG Fc includes the Leu residue at position 235 of IgG Fc. Thus, the Fc receptor binding site can be destroyed by mutating or deleting Leu 235. For example, substitution of Glu for Leu 235 inhibits the ability of the Fc region to bind the high affinity Fc receptor. The murine C'1q binding site can be functionally destroyed by mutating or deleting the Glu 318, Lys 320, and Lys 322 residues of IgG. For example, substitution of Ala residues for Glu 318, Lys 320, and Lys 322 renders IgG1 Fc unable to direct antibody-dependent complement lysis. In contrast, a lytic IgG Fc region has a high affinity Fc receptor binding site and a C'1q binding site. The high affinity Fc receptor binding site includes the Leu residue at position 235 of IgG Fc, and the C'1q binding site includes the Glu 318, Lys 320, and Lys 322 residues of IgG1. Lytic IgG Fc has wild-type residues or conservative amino acid substitutions at these sites. Lytic IgG Fc can target cells for antibody-dependent cellular cytotoxicity or complement directed cytolysis (CDC). Appropriate mutations for human IgG are also known (see, e.g., Morrison et al., *The Immunologist* 2:119-124, 1994; and Brekke et al., *The Immunologist* 2:125, 1994).

In other instances, the chimeric polypeptide may include the mutant IL-15 polypeptide and a polypeptide that functions as an antigenic tag, such as a FLAG sequence. FLAG sequences are recognized by biotinylated, highly specific, anti-FLAG antibodies, as described herein (see also Blanar et al., *Science* 256:1014, 1992;

- 11 -

groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

5 The mutations described above can be in the carboxy-terminal domain of the cytokine, which is believed to bind the IL-2R γ subunit. It is also possible that one or more mutations can be within the IL-2R β binding domain.

10 In a related aspect, the invention features a chimeric polypeptide that includes a mutant IL-15 polypeptide and a heterologous polypeptide (i.e., a polypeptide that is not IL-15 or a mutant thereof). The heterologous polypeptide can increase the circulating 15 half-life of the chimeric polypeptide *in vivo*. The polypeptide that increases the circulating half-life may be a serum albumin, such as human serum albumin, or the Fc region of the IgG subclass of antibodies that lacks the IgG heavy chain variable region. The Fc region may 20 include a mutation that inhibits complement fixation and Fc receptor binding, or it may be lytic, i.e., able to bind complement or to lyse cells via another mechanism, such as antibody-dependent complement lysis (ADCC; 08/355,502 filed 12/12/94).

25 The "Fc region" can be a naturally-occurring or synthetic polypeptide that is homologous to the IgG C-terminal domain produced by digestion of IgG with papain. IgG Fc has a molecular weight of approximately 50 kDa. The polypeptides of the invention can include 30 the entire Fc region, or a smaller portion that retains the ability to extend the circulating half-life of a chimeric polypeptide of which it is a part. In addition, full-length or fragmented Fc regions can be variants of the wild-type molecule. This is, they can contain 35 mutations that may or may not affect the function of the

- 14 -

facilitate purification of bacterially expressed protein, or to a hemagglutinin tag to facilitate purification of protein expressed in eukaryotic cells.

The techniques that are required to make mutant IL-15 polypeptides are routine in the art, and can be performed without resort to undue experimentation by artisans of ordinary skill. For example, a mutation that consists of a substitution of one or more of the amino acid residues in IL-15 can be created using the PCR-assisted mutagenesis technique described herein for the creation of the mutant IL-15 polypeptide in which glutamine residues at positions 149 and 156 were changed to aspartic acid residues. Mutations that consists of deletions or additions of amino acid residues to an IL-15 polypeptide can also be made with standard recombinant techniques. In the event of a deletion or addition, the nucleic acid molecule encoding IL-15 is simply digested with an appropriate restriction endonuclease. The resulting fragment can either be expressed directly, or manipulated further, for example, by ligating it to a second fragment. The ligation may be facilitated if the two ends of the nucleic acid molecules contain complementary nucleotides that overlap one another, but blunt-ended fragments can also be ligated.

In addition to generating mutant polypeptides via expression of nucleic acid molecules that have been altered by recombinant molecular biological techniques, mutant polypeptides can be chemically synthesized. Chemically synthesized polypeptides are routinely generated by those of skill in the art.

- 13 -

LeClair et al., Proc. Natl. Acad. Sci. USA 89:8145, 1992).

Chimeric polypeptides can be constructed using no more than conventional molecular biological techniques, 5 which are well within the ability of those of ordinary skill in the art to perform.

As used herein, the terms "protein" and "polypeptide" both refer to any chain of amino acid residues, regardless of length or post-translational 10 modification (e.g., glycosylation or phosphorylation). The proteins or polypeptides of the invention are "substantially pure," meaning that they are at least 60% by weight (dry weight) the polypeptide of interest, e.g., a polypeptide containing the mutant IL-15 amino acid 15 sequence. Preferably, the polypeptide is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, the polypeptide of interest. Purity can be measured by any appropriate standard method, e.g., column chromatography, polyacrylamide gel 20 electrophoresis, or HPLC analysis.

Although the polypeptide can be naturally occurring, it is likely that the polypeptides used in the practice of the instant invention will be synthetic, or produced by expression of a recombinant nucleic acid 25 molecule. Polypeptides that are derived from eukaryotic organisms or synthesized in *E. coli*, or other prokaryotes, and polypeptides that are chemically synthesized will be substantially free from their naturally associated components. In the event the 30 polypeptide is a chimera, it can be encoded by a hybrid nucleic acid molecule (such as those discussed further below) containing one sequence that encodes all or part of the mutant IL-15, and a second sequence that encodes all or part of a second gene. For example, the mutant 35 IL-15 polypeptide may be fused to a hexa-histidine tag to

- 16 -

techniques. Once the heart is grafted in place and warmed to 37°C with Ringer's lactate solution, normal sinus rhythm will resume. Function of the transplanted heart can be assessed frequently by palpation of 5 ventricular contractions through the abdominal wall. Rejection is defined as the cessation of myocardial contractions, which can be confirmed by under anesthesia. Mutant IL-15 polypeptides would be considered effective in reducing organ rejection if hosts that received 10 injections, for example, of the mutant IL-15 polypeptide experienced longer heart engraftment than the untreated hosts.

Skin Grafting

The effectiveness of mutant IL-15 polypeptides can 15 also be assessed following a skin graft. To perform skin grafts on a rodent, a donor animal is anesthetized and the full thickness skin is removed from a part of the tail. The recipient animal is also anesthetized, and a graft bed is prepared by removing a patch of skin from 20 the shaved flank. Generally, the patch is approximately 0.5 x 0.5 cm². The skin from the donor is shaped to fit the graft bed, positioned, covered with gauze, and bandaged. The grafts can be inspected daily beginning on the sixth post-operative day, and are considered rejected 25 when more than half of the transplanted epithelium appears to be non-viable. Mutant IL-15 polypeptides would be considered effective in reducing rejection of the skin grafts if hosts that received injections, for example, of the mutant IL-15 polypeptide tolerated the 30 graft longer than the untreated hosts.

Islet Allograft Model

DBA/2J islet cell allografts can be transplanted into rodents, such as 6-8 week-old IL-4^{+/+} and IL-4^{-/-} mice

- 15 -

Procedures for Screening Mutant IL-15 Polypeptides

In addition to testing a candidate mutant IL-15 polypeptide in the *in vitro* assays described in the examples below, the following procedures can be employed
5 to determine whether a mutant IL-15 polypeptide is capable of functioning as an antagonist of IL-15 *in vivo*.

Transplantation Paradigms

In order to determine whether a polypeptide is capable of functioning as an immunosuppressant, it can be
10 administered, either directly or by genetic therapy, in the context of well-established transplantation paradigms.

A putative immunosuppressing polypeptide, or a nucleic acid molecule encoding it, could be systemically
15 or locally administered by standard means to any conventional laboratory animal, such as a rat, mouse, rabbit, guinea pig, or dog, before an allogeneic or xenogeneic skin graft, organ transplant, or cell implantation is performed on the animal. Strains of mice
20 such as C57Bl-10, B10.BR, and B10.AKM (Jackson Laboratory, Bar Harbor, ME), which have the same genetic background but are mismatched for the H-2 locus, are well suited for assessing various organ grafts.

Heart Transplantation

25 A method for performing cardiac grafts by anastomosis of the donor heart to the great vessels in the abdomen of the host was first published by Ono et al. (J. Thorac. Cardiovasc. Surg. 57:225, 1969; see also Corry et al., Transplantation 16:343, 1973). According
30 to this surgical procedure, the aorta of a donor heart is anastomosed to the abdominal aorta of the host, and the pulmonary artery of the donor heart is anastomosed to the adjacent vena cava using standard microvascular

- 18 -

their F₁ hybrids. These animals can be crossed in order to study particular aspects of the rheumatic disease process; progeny of the NZB strain develop severe lupus glomerulonephritis when crossed with NZW mice

5 (Bielschowsky et al., *Proc. Univ. Otago Med. Sch.* 37:9, 1959; see also *Fundamental Immunology*, Paul, Ed., Raven Press, New York, NY, 1989). Similarly, a shift to lethal nephritis is seen in the progeny of NBZ X SWR matings (Data et al., *Nature* 263:412, 1976). The histological 10 appearance of renal lesions in SNF₁ mice has been well characterized (Eastcott et al., *J. Immunol.* 131:2232, 1983; see also *Fundamental Immunology*, *supra*). Therefore, the general health of the animal as well as the histological appearance of renal tissue can be used 15 to determine whether the administration of a mutant IL-15 polypeptide can effectively suppress the immune response in an animal model of SLE.

One of the MRL strains of mice that develops SLE, MRL-1pr/1pr, also develops a form of arthritis that 20 resembles rheumatoid arthritis in humans (Theofilopoulos et al., *Adv. Immunol.* 37:269, 1985). Alternatively, an experimental arthritis can be induced in rodents by injecting rat type II collagen (2 mg/ml) mixed 1:1 in Freund's complete adjuvant (100 µl total) into the base 25 of the tail. Arthritis develops 2-3 weeks after immunization. The ability of nucleic acid molecules encoding mutant IL-15 polypeptides to combat the arthritic condition can be assessed by targeting the nucleic acid molecules to T lymphocytes. One way to 30 target T lymphocytes is the following: spleen cell suspensions are prepared 2-3 days after the onset of arthritis and incubated with collagen (100 µg/ml) for 48 hours to induce proliferation of collagen-activated T cells. During this time, the cells are transduced with 35 a vector encoding the polypeptide of interest. As a

- 17 -

rendered diabetic by a single intraperitoneal injection of streptozotocin (225 mg/kg; Sigma Chemical Co., St. Louis, MO). As a control, syngeneic IL-4^{-/-} islet cell grafts can be transplanted into diabetic IL-4^{-/-} mice.

5 Islet cell transplantation can be performed by following published protocols (for example, see Gotoh et al., *Transplantation* 42:387, 1986). Briefly, donor pancreata are perfused *in situ* with type IV collagenase (2 mg/ml; Worthington Biochemical Corp., Freehold, NJ). After a

10 40 minute digestion period at 37°C, the islets are isolated on a discontinuous Ficoll gradient. Subsequently, 300-400 islets are transplanted under the renal capsule of each recipient. Allograft function can be followed by serial blood glucose measurements (Accu-

15 Check II™; Boehringer, Mannheim, Germany). Primary graft function is defined as a blood glucose level under 11.1 mmol/l on day 3 post-transplantation, and graft rejection is defined as a rise in blood glucose exceeding 16.5 mmol/l (on reaches of at least 2 successive days)

20 following a period of primary graft function.

2.2.2 Models of Autoimmune Disease

25 Models of autoimmune disease provide another means to assess polypeptides *in vivo*. These models are well known to skilled artisans, and can be used to determine whether a given mutant IL-15 polypeptide is an immunosuppressant that would be therapeutically useful in treating a specific autoimmune disease when delivered either directly or via genetic therapy.

30 Animals include rheumatic diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE), type I diabetes, and autoimmune diseases of the thyroid, gut, and central nervous system. For example, animal models of SLE include MRL mice, BXSB mice, and NZB mice and

- 20 -

induced by a single injection of brain or spinal cord tissue with adjuvant in many different laboratory animals, including rodents and primates. This model, referred to as experimental allergic encephalomyelitis 5 (EAE) is T cell mediated. Similarly, experimentally induced myasthenia gravis can be produced by a single injection of acetylcholine receptor with adjuvants (Lennon et al., Ann. N.Y. Acad. Sci. 274:283, 1976).

Autoimmune diseases of the gut can be modeled in 10 IL-2 or IL-10 "knock out" mice, or in mice that receive enemas containing bovine serum albumin.

Nucleic Acid Molecules Encoding Mutant IL-15

The mutant IL-15 polypeptide, either alone or as a part of a chimeric polypeptide, such as those described 15 above, can be obtained by expression of a nucleic acid molecule. Thus, nucleic acid molecules encoding polypeptides containing a mutant IL-15 are considered within the scope of the invention. Just as the mutant 20 IL-15 polypeptides can be described in terms of their identity with wild-type IL-15 polypeptides, the nucleic acid molecules encoding them will necessarily have a certain identity with those that encode wild-type IL-15. For example, the nucleic acid molecule encoding a mutant 25 IL-15 polypeptide can be at least 65%, preferably at least 75%, more preferably at least 85%, and most preferably at least 95% (e.g., 99%) identical to the nucleic acid encoding wild-type IL-15. For nucleic acids, the length of the sequences compared will generally be at least 50 nucleotides, preferably at least 30 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

The nucleic acid molecules of the invention can contain naturally occurring sequences, or sequences that differ from those that occur naturally, but, due to the

- 19 -

control, parallel cultures are grown but not transduced or, transduced with the "empty" vector. The cells are then injected intraperitoneally (5×10^7 cells/animal). The effectiveness of the treatment is assessed by 5 following the disease symptoms during the subsequent 2 weeks, as described by Chernajovsky et al. (*Gene Therapy* 2:731-735, 1995). Lesser symptoms, compared to control, indicate that the peptide of interest, and the 10 nucleic acid molecule encoding it, function as an immunosuppressant potentially useful in the treatment of autoimmune disease.

The ability of a mutant IL-15 polypeptide to suppress the immune response in the case of Type I diabetes can be tested in the BB rat strain, which was 15 developed from a commercial colony of Wistar rats at the Bio-Breeding Laboratories in Ottawa. These rats spontaneously develop autoantibodies against islet cells and insulin, just as occurs with human Type I diabetes. Alternatively, NOD (non-obese diabetic) mice can be used 20 as a model system.

Autoimmune diseases of the thyroid have been modeled in the chicken. Obese strain (OS) chickens consistently develop spontaneous autoimmune thyroiditis resembling Hashimoto's disease (Cole et al., *Science* 25 160:1357; 1968). Approximately 15% of these birds produce autoantibodies to parietal cells of the stomach, just as in the human counterpart of autoimmune thyroiditis. The manifestations of the disease in OS chickens, which could be monitored in the course of 30 mutant IL-15 polypeptide treatment, include body size, fat deposit, serum lipids, cold sensitivity, and infertility.

Models of autoimmune disease in the central nervous system (CNS) can be experimentally induced. An 35 inflammation of the CNS, which leads to paralysis, can be

- 22 -

As described above, the mutant IL-15 polypeptide of the invention may exist as a part of a chimeric polypeptide. In addition to, or in place of, the heterologous polypeptides described above, a nucleic acid molecule of the invention can contain sequences encoding a "marker" or "reporter." Examples of marker or reporter genes include β -lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo^r, G418^r), 10 dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), lacZ (encoding β -galactosidase), and xanthine guanine phosphoribosyltransferase (XGPRT). As with many of the standard procedures associated with the practice of the invention, skilled artisans will be aware of additional useful reagents, for example, of additional sequences that can serve the function of a marker or reporter.

The nucleic acid molecules of the invention can be obtained by introducing a mutation into IL-15-encoding 20 DNA obtained from any biological cell, such as the cell of a mammal. Thus, the nucleic acids of the invention can be those of a mouse, rat, guinea pig, cow, sheep, horse, pig, rabbit, monkey, baboon, dog, or cat. Preferably, the nucleic acid molecules will be those of a 25 human.

Expression of Mutant IL-15 Gene Products

The nucleic acid molecules described above can be contained within a vector that is capable of directing their expression in, for example, a cell that has been 30 transduced with the vector. Accordingly, in addition to mutant IL-15 polypeptides, expression vectors containing a nucleic acid molecule encoding a mutant IL-15 polypeptide and cells transfected with these vectors are among the preferred embodiments.

- 21 -

degeneracy of the genetic code, encode the same polypeptide. These nucleic acid molecules can consist of RNA or DNA (for example, genomic DNA, cDNA, or synthetic DNA, such as that produced by phosphoramidite-based synthesis), or combinations or modifications of the nucleotides within these types of nucleic acids. In addition, the nucleic acid molecules can be double-stranded or single-stranded (i.e., either a sense or an antisense strand).

10 The nucleic acid molecules of the invention are referred to as "isolated" because they are separated from either the 5' or the 3' coding sequence with which they are immediately contiguous in the naturally occurring genome of an organism. Thus, the nucleic acid molecules 15 are not limited to sequences that encode polypeptides; some or all of the non-coding sequences that lie upstream or downstream from a coding sequence can also be included. Those of ordinary skill in the art of molecular biology are familiar with routine procedures 20 for isolating nucleic acid molecules. They can, for example, be generated by treatment of genomic DNA with restriction endonucleases, or by performance of the polymerase chain reaction (PCR). In the event the nucleic acid molecule is a ribonucleic acid (RNA), 25 molecules can be produced by *in vitro* transcription.

The isolated nucleic acid molecules of the invention can include fragments not found as such in the natural state. Thus, the invention encompasses recombinant molecules, such as those in which a nucleic acid sequence (for example, a sequence encoding a mutant IL-15) is incorporated into a vector (for example, a plasmid or viral vector) or into the genome of a heterologous cell (or the genome of a homologous cell, at a position other than the natural chromosomal location).

- 24 -

(SV40), and bovine papilloma virus vectors (see, for example, Gluzman (Ed.), *Eukaryotic Viral Vectors*, CSH Laboratory Press, Cold Spring Harbor, New York).

Prokaryotic or eukaryotic cells that contain and 5 express a nucleic acid molecule that encodes a mutant IL-15 polypeptide are also features of the invention. A cell of the invention is a transfected cell, i.e., a cell into which a nucleic acid molecule, for example a nucleic acid molecule encoding a mutant IL-15 polypeptide, has 10 been introduced by means of recombinant DNA techniques.

The progeny of such a cell are also considered within the scope of the invention. The precise components of the expression system are not critical. For example, a mutant IL-15 polypeptide can be produced in a prokaryotic 15 host, such as the bacterium *E. coli*, or in a eukaryotic host, such as an insect cell (for example, Sf21 cells), or mammalian cells (e.g., COS cells, NIH 3T3 cells, or HeLa cells). These cells are available from many sources, including the American Type Culture Collection 20 (Rockville, MD). In selecting an expression system, it matters only that the components are compatible with one another. Artisans or ordinary skill are able to make such a determination. Furthermore, if guidance is required in selecting an expression system, skilled 25 artisans may consult Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley and Sons, New York, NY, 1993) and Pouwels et al. (*Cloning Vectors: A Laboratory Manual*, 1985, Suppl. 1987).

The expressed polypeptides can be purified from 30 the expression system using routine biochemical procedures, and can be used as diagnostic tools or as therapeutic agents, as described below.

- 23 -

Vectors suitable for use in the present invention include T7-based vectors for use in bacteria (see, for example, Rosenberg et al., *Gene* 56:125, 1987), the pMSXND expression vector for use in mammalian cells (Lee and 5 Nathans, *J. Biol. Chem.* 263:3521, 1988), and baculovirus-derived vectors (for example the expression vector pBacPAK9 from Clontech, Palo Alto, CA) for use in insect cells. The nucleic acid inserts, which encode the polypeptide of interest in such vectors, can be operably 10 linked to a promoter, which is selected based on, for example, the cell type in which expression is sought. For example, a T7 promoter can be used in bacteria, a polyhedrin promoter can be used in insect cells, and a 15 cytomegalovirus or metallothionein promoter can be used in mammalian cells. Also, in the case of higher eukaryotes, tissue-specific and cell type-specific promoters are widely available. These promoters are so named for their ability to direct expression of a nucleic acid molecule in a given tissue or cell type within the 20 body. Skilled artisans are well aware of numerous promoters and other regulatory elements which can be used to direct expression of nucleic acids.

In addition to sequences that facilitate transcription of the inserted nucleic acid molecule, 25 vectors can contain origins of replication, and other genes that encode a selectable marker. For example, the neomycin-resistance (*neo*^r) gene imparts G418 resistance to cells in which it is expressed, and thus permits phenotypic selection of the transfected cells. Those of 30 skill in the art can readily determine whether a given regulatory element or selectable marker is suitable for use in a particular experimental context.

Viral vectors that can be used in the invention include, for example, retroviral, adenoviral, and 35 adeno-associated vectors, herpes virus, simian virus 40

- 26 -

polypeptide, including those that are a part of the chimeric polypeptides described above. This method may be used to treat a patient who is suffering from an autoimmune disease, including but not limited to the following: (1) a rheumatic disease such as rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, scleroderma, mixed connective tissue disease, dermatomyositis, polymyositis, Reiter's syndrome or Behcet's disease (2) type II diabetes (3) an autoimmune disease of the thyroid, such as Hashimoto's thyroiditis or Graves' Disease (4) an autoimmune disease of the central nervous system, such as multiple sclerosis, myasthenia gravis, or encephalomyelitis (5) a variety of pemphigus, such as pemphigus vulgaris, pemphigus vegetans, pemphigus foliaceus, Senear-Usher syndrome, or Brazilian pemphigus, (6) psoriasis, and (7) inflammatory bowel disease (e.g., ulcerative colitis or Crohn's Disease). The administration of the mutant IL-15 polypeptide of the invention may also be useful in the treatment of acquired immune deficiency syndrome (AIDS). Similarly, the method may be used to treat a patient who has received a transplant of biological materials, such as an organ, tissue, or cell transplant. In addition, patients who have received a vascular injury would benefit from this method.

Induction of a Lytic Response

Through the administration of a lytic form of a mutant IL-15 chimeric polypeptide, it is possible to selectively kill autoreactive or "transplant destructive" immune cells without massive destruction of normal T cells. Accordingly, the invention features a method of killing autoreactive or "transplant destructive" immune cells or any malignant cell that expresses the IL-15 receptor in vivo. The method is carried out by

- 25 -

Mutant IL-15 Polypeptides in Diagnosis

The polypeptide of the invention can be used to diagnose a patient as having a disease amenable to treatment with an IL-15 antagonist. According to this 5 method, a sample of tissue is obtained from the patient and exposed to an antigenically-tagged mutant IL-15 polypeptide. The sample may be any biological sample, such as a blood, urine, serum, or plasma sample. In 10 addition, the sample may be a tissue sample (e.g., biopsy tissue), or an effusion obtained from a joint (e.g. synovial fluid), from the abdominal cavity (e.g., ascites fluid), from the chest (e.g., pleural fluid), from the central nervous system (e.g., cerebral spinal fluid), or from the eye (e.g., aqueous humor). The sample may also 15 consist of cultured cells that were originally obtained from a patient (e.g. peripheral blood mononuclear cells). It is expected that the sample will be obtained from a mammal, and preferably, that the mammal will be a human patient. If the sample contains cells that are bound by 20 the polypeptide described (i.e., an antigenically-tagged mutant IL-15 polypeptide), it is highly likely that they would be bound by a mutant IL-15 polypeptide *in vivo* and thereby inhibited from proliferating, or even destroyed, *in vivo*. The presenting symptoms of candidate patients 25 for such testing and the relevant tissues to be sampled given a particular set of symptoms are known to those skilled in the field of immunology.

Modulation of the Immune Response

Also featured in the invention is a method of 30 suppressing the immune response in a patient by administering a dose of mutant IL-15 that is sufficient to competitively bind the IL-15 receptor complex, and thereby modulate IL-15 dependent immune responses. The polypeptide administered can be a mutant IL-15

oral administration. It is expected that the intravenous route will be preferred for the administration of mutant IL-15 polypeptides. The subcutaneous route may also be used frequently as the subcutaneous tissue provides a 5 stable environment for the polypeptide, from which it can be slowly released.

The polypeptides of the invention are less likely to be immunogenic than many therapeutic polypeptides because they can differ from a wild-type polypeptide by 10 only a few amino acid residues. In addition, the mutant IL-15 polypeptides are targeted to cells that express abundant receptors to which they can bind with high affinity.

It is well known in the medical arts that dosages 15 for any one patient depend on many factors, including the general health, sex, weight, body surface area, and age of the patient, as well as the particular compound to be administered, the time and route of administration, and other drugs being administered concurrently. Dosages for 20 the polypeptide of the invention will vary, but can, when administered intravenously, be given in doses of approximately 0.01 mg to 100 mg/ml blood volume. A dosage can be administered one or more times per day, if necessary, and treatment can be continued for prolonged 25 periods of time, up to and including the lifetime of the patient being treated. If a polypeptide of the invention is administered subcutaneously, the dosage can be reduced, and/or the polypeptide can be administered less frequently. Determination of correct dosage for a given 30 application is well within the abilities of one of ordinary skill in the art of pharmacology.

- 27 -

administering to a patient an amount of mutant IL-15 linked to a polypeptide that is sufficient to activate the complement system, lyse cells by the ADCC mechanism, or otherwise kill cells expressing the wild-type IL-15 receptor complex. This method can be used to treat patients who have IL-15R⁺ leukemia, lymphoma, or other IL-15R⁺ malignant diseases, such as colon cancer.

Formulations for Use and Routes of Administration

In therapeutic applications, the polypeptide may be administered with a physiologically-acceptable carrier, such as physiological saline. The therapeutic compositions of the invention can also contain a carrier or excipient, many of which are known to skilled artisans. Excipients which can be used include buffers (for example, citrate buffer, phosphate buffer, acetate buffer and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (for example, serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol. The polypeptides of the invention can be formulated in various ways, according to the corresponding route of administration. For example, liquid solutions can be made for ingestion or injection; gels or powders can be made for ingestion, inhalation, or topical application.

Methods for making such formulations are well known and can be found in, for example, "Remington's Pharmaceutical Sciences".

Routes of administration are also well known to skilled pharmacologists and physicians and include intraperitoneal, intramuscular, subcutaneous, and intravenous administration. Additional routes include intracranial (e.g., intracisternal or intraventricular), intraorbital, ophthalmic, intracapsular, intraspinal, intraperitoneal, transmucosal, topical, subcutaneous, and

- 30 -

covalently bound to the 18 amino acid FLAG-HMK-sequence (FLAG-HMK-IL-15). FLAG sequences are recognized by biotinylated, highly specific anti-FLAG antibodies (Blanar et al., *Science* 256:1014, 1992); LeClair et al., 5 *Proc. Natl. Acad. Sci. USA* 89:8145, 1992) while HMK (Heart Muscle Kinase recognition site) sequences allow introduction of radioactive label [³²P] into the molecule (Blanar et al., *supra*, LeClair et al., *supra*).

For the construction of the plasmid FLAG-HMK-IL-15, a 322 bp cDNA fragment encoding mature IL-15 protein was amplified by PCR utilizing synthetic oligonucleotides [sense 5' -GGAATTCAACTGGGTGAATGTAATA-3' (SEQ ID NO:1; EcoRI site (underlined) plus bases 145-162); antisense 5' -CGGGATCCTCAAGAAGTGTGATGAA-3' (SEQ ID NO:2; BamHI site 15¹²[underlined] plus bases 472-489)]. The template DNA was obtained from PHA-activated human PBMCs. The PCR product was purified, digested with EcoRI and BamHI, and cloned into the pAR(DRI)59/60 plasmid digested with EcoRI-BamHI as described (Blanar et al., *Science* 256:1014, 1992; 10 LeClair et al., *Proc. Natl. Acad. Sci. USA* 89:8145, 1992). The backbone of the pAR(DRI)59/60 plasmid contains in-frame sequences encoding the FLAG and HMK recognition peptide sequences (Blanar et al., *Science* 256:1014, 1992; LeClair et al., *Proc. Natl. Acad. Sci. 15 USA* 89:8145, 1992).

Expression and Purification of FLAG-HMK-IL-15 Fusion Protein

The IL-15-related fusion construct, FLAG-HMK-IL-15, was expressed in BL-21 strain *E. coli* and affinity 20 purified with anti-FLAG coated beads as described (Blanar et al., *Science* 256:1014, 1992; LeClair et al., *Proc. Natl. Acad. Sci. USA* 89:8145, 1992). The fusion protein was eluted from affinity columns after extensive washing 25 with 0.1 M glycine (pH 3.0). The eluate containing FLAG-HMK-IL-15 was dialyzed against a buffer containing 50 mM

- 29 -

EXAMPLES

Reagents

The following reagents were used in the studies described herein: recombinant human IL-2 was obtained 5 from Hoffman-La Roche (Nutley, NJ); rapamycin was obtained from Wyeth-Ayerst (Princeton, NJ); cyclosporine-A (CsA) was obtained from Sandoz (East Hanover, NJ); RPMI-1640 and fetal calf serum (FCS) were obtained from BioWittaker (Walkersville, MD); penicillin, 10 streptomycin, G418, and strepavidin-RED670 were obtained from Gibco-BRL (Gaithersburg, MD); dexamethasone, PHA, lysozyme, Nonidet P-40, NaCl, HEPES, and PMSF were obtained from Sigma (St. Louis, MO); Ficoll-Hypaque was obtained from Pharmacia Biotech (Uppsala, Sweden); 15 recombinant human IL-15 and anti-human IL-15 Ab were obtained from PeproTech (Rocky Hill, NJ); anti-FLAG Ab and anti-FLAG-affinity beads were obtained from International Biotechnologies, Inc. (Kodak, New Haven, CT); pRcCMV was obtained from InVitrogen Corporation (San 20 Diego, CA); genistein was obtained from ICN Biomedicals (Irvine, CA); disuccinimidyl suberate (DSS) was obtained from Pierce (Rockford, IL); restriction endonucleases were obtained from New England Biolabs (Beverly, MA); [³H]TdR was obtained from New England Nuclear (Boston, 25 MA); and fluorescent dye conjugated antibodies CD25-PE, CD14-PE, CD16-PE, CD122-PE, CD4-FITC, CD8-FITC, IgG1-PE or IgG1-FITC were obtained from Beckton/Dickinson (San Jose, CA). FLAG peptide was synthesized in the Peptide Synthesis Facility at Harvard Medical School.

30

Production of FLAG-HMK-IL-15 Fusion Protein

To study the cellular pattern of human IL-15 receptor expression, a plasmid that could be used to express an IL-15 fusion protein was constructed. The plasmid encodes an IL-15 polypeptide having an N-terminus

- 32 -

For studies involving rapamycin or genistein, human PBMCs (4×10^5 /well) were plated in U-bottomed 96-well tissue culture plates (NUNC; Naperville, IL) with rapamycin (10 ng/ml), genistein (10 μ g/ml) or media 5 alone, and cultured for 3 days at 37°C and 5% CO₂. Cell viability was tested utilizing the standard trypan blue exclusion method (Current Protocols in Immunology, Vol. 3, ed. R. Coico, New York: John Wiley & Sons, 1995).

BAF-BO3 Cells

10 The BAF-BO3 cell line is an IL-3-dependent, murine pro-B cell line. A BAF-BO3 cell line that was pre-selected for high expression of the IL-2R α subunit was kindly provided by T. Taniguchi (Osaka, Japan; Hatakeyama et al., 1989, Cell 59:837). BAF-BO3 cells were 15 transfected with vector control (pRcCMV-0) or cDNA encoding the human IL-2R β subunit cloned into pRcCMV (pRcCMV-IL-2R β), by electroporation at 350 volts and 500 μ Farads using 20 μ g of *ScaI* linearized plasmid. Transfected cells were selected in the presence of 1 20 mg/ml G418 and tested for the expression of the IL-2R β subunit by fluorescence activated cell sorting (FACS) analysis as described (Maslinski et al., *Int J Immunol.* 4:509, 1992; Hatakeyama et al., *Cell* 59:837, 1989).

25 BAF-BO3 cells were maintained at 37°C with 5% CO₂, in RPMI-1640 medium supplemented with 10% FCS, penicillin (0.5 U/ml), streptomycin (0.5 μ g/ml), and 5% (V/V) IL-3 rich WEHI cell supernatant (Hatakeyama et al., *Cell* 59:837, 1989).

30 To test mutant polypeptides, a cytotoxic T lymphocyte cell line, CTLL-2, can also be used. This cell line is available from the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852-1776).

- 31 -

Tris (pH 7.4) and 0.1 M NaCl for 18 hours at 4°C, filtered through a 0.2 µm membrane, and stored at -20°C.

Analysis of FLAG-HMK-IL-15 Fusion Protein

The authenticity of FLAG-HMK-IL-15 fusion protein was confirmed as follows. The proteins present in eluates from the affinity column were separated by SDS-PAGE, transferred to PVDF membranes, and stained with Coomassie blue (Fig. 1, lane a). The presence of FLAG sequences was specifically examined using anti-FLAG Ab (Fig. 1, lane b), and the presence of hIL-15 sequences was specifically examined using anti-hIL-15 Ab (Fig. 1, lane c). A 15 kDa protein, whose mass corresponds to the expected size of FLAG-HMK-IL-15 protein, was identified in each of these analyses. To confirm that the fusion protein contains the HMK recognition site, eluates from the affinity column were incubated in the presence of enzymatically active HMK and radioactive [³²P]-γ-ATP, followed by separation of proteins by SDS-PAGE.

Autoradiography again identified a single radiolabeled 15 kDa band that co-migrates with the FLAG-HMK-IL-15 (Fig. 1, lane d). Thus, the 15 kDa protein contains all three elements of the designed fusion protein: FLAG sequence, HMK sequence, and hIL-15 sequence.

Cell Culture

Human PBMCs

Human peripheral blood mononuclear cells (PBMCs) were obtained from leukapheresis preparations from healthy donors and isolated by Ficoll-Hypaque density gradient centrifugation according to standard protocols. PBMCs were maintained at 37°C in an atmosphere containing 5% CO₂ in RPMI-1640 medium supplemented with 10% FCS, penicillin (0.5 U/ml), and streptomycin (0.5 µg/ml).

- 34 -

subunits, and which, therefore, proliferate in response to exogenously added human IL-2 or IL-15 (Giri et al., *EMBO J.* 13:2822, 1994). Cells were washed twice to remove the medium containing the growth factor IL-3, and 5 starved for 16 hours in RPMI-1640 medium supplemented with 10% FCS, penicillin (0.5 U/ml), and streptomycin (0.5 μ g/ml). Cells were then plated at 2×10^4 cells/well, stimulated with FLAG-HMK-IL-15, and cultured for 72 hours at 37°C in an atmosphere containing 5% CO₂. 10 This was followed by a 14-hour "pulse" with 1 μ Ci of [³H]-TdR. The BAF-BO3 cells were then harvested and cell-associated radioactivity was measured by scintillation counting as described above.

15 1.1a-5a Cross-linking Activated PBMCs Cultured with FLAG-HMK-IL-15

20 After 72 hours in culture, PHA-activated PBMCs were washed twice in cold phosphate-buffered saline (PBS), and 10⁷ cells/ml were incubated for 30 minutes at 4°C in 400 μ l of RPMI-1640 medium supplemented with 25 mM HEPES (pH 7.4), 10% FCS, penicillin (0.5 U/ml), and streptomycin (0.5 μ g/ml). Supplemented RPMI-1640 medium (20 μ l) containing 1 μ g of recombinant human IL-15 as a control for non-specific binding or medium alone was then added, and the incubation was continued for 15 minutes 25 before 20 μ l of medium containing [³²P]-labeled FLAG-HMK-IL-15 (1 ng, 500,000 cpm) was added to each sample. After further incubation for 1 hour at 4°C, the cells were washed with PBS and resuspended in 250 μ l of PBS. 30 Disuccinimidyl suberate (DDS, 1 mM), a cross-linker, was added, and cross-linking was performed as described by Tsude et al. (*Proc. Natl. Acad. Sci. USA* 83:9694, 1986). The cross-linked cells were collected by centrifugation, washed, solubilized with extraction buffer (2% Nonidet P-40, 0.14 M NaCl, 25 mM HEPES (pH 7.4), 1 mM phenyl-35 methylsulfonyl fluoride) and centrifuged. Proteins

- 33 -

Cellular Proliferation Assays

Human PBMCs

Peripheral blood mononuclear cells (1×10^6 /ml) were stimulated with PHA (2 μ g/ml) for 72 hours at 37°C in RPMI-1640 medium supplemented with 10% FCS, penicillin, and streptomycin, as described above. To investigate the effect of immunosuppressive drugs, aliquots of cells (10^6 PBMCs/ml) were preincubated with cyclosporin-A (CsA; 150 ng/ml), rapamycin (5 ng/ml), or dexamethasone (0.1 ng/ml) for 20 minutes before the addition of PHA.

In some experiments, cells prepared as described above were washed after 72 hours in culture, plated in U-bottomed 96-well tissue culture plates at 2×10^5 PBMCs per well (NUNC, Naperville, IL), and then re-stimulated with one of the following reagents: PHA (2 μ g/ml), FLAG peptide (10⁻⁴ M), IL-2 (100 U/ml), or FLAG-HMK-IL-15 (100 μ g/ml) for 72 hours. Cells were "pulsed" for 4 hours with μ Ci of [³H]TdR (New England Nuclear, Boston, MA), harvested onto Whatman 934-AH glass microfiber filters, and hypotonically lysed using a PHD Cell Harvester (Cambridge Technology, Inc., Cambridge, MA). Cell associated [³H]-TdR was measured using a Beckman LS 2800 scintillation counter (Beckman, Fullerton, CA).

BAF-BO3 cells

IL-3-dependent BAF-BO3 cells, which constitutively express the IL-2R γ subunit, were transfected with a control pRcCMV construct (pRcCMV-0) or a pRcCMV construct containing cDNA encoding the full-length human IL-2R β subunit and the neor gene (pRcCMV-IL-2R β). G418^r resistant clones were then selected. Cells that were selected in this way were used to test the biological activity of FLAG-HMK-IL-15. This strategy has been used to identify cells transfected with cDNA encoding full-length IL-2R β .

- 36 -

molecular weight of FLAG-HMK-IL-15 (15 kDa) and the human IL-15R α subunit (60-65 kDa; Fig. 2, lane 1). The identity of this band as the IL-15R α subunit was confirmed by cross-linking experiments conducted in the 5 presence of a molar excess of hIL-15. Under these conditions, we failed to detect the radiolabeled 15 kDa band (Fig. 2, lane 2). Thus, the conformation of [32 P] - FLAG-HMK-IL-15 fusion proteins allows site specific binding to the 60-65 kDa IL-15R α subunit expressed on the 10 surface of mitogen-activated PBMCS.

FLAG-HMK-IL-15 is a Biologically Active Growth Factor that Requires Expression of IL-2R β

In the next series of experiments, the FLAG-HMK-IL-15 fusion protein was tested in order to determine 15 whether it could function as a biologically active growth factor. PHA-activated human PBMCS proliferate in response to either FLAG-HMK-IL-15 or human recombinant IL-2, as detected via the [3 H] - TdR incorporation assay described above. A FLAG peptide lacking the IL-15 20 sequence does not stimulate cell proliferation (Fig. 3a). As does IL-2, the FLAG-HMK-IL-15 fusion protein stimulates proliferation of IL-2R γ BAF-BO3 cell transfectants that express the IL-2R β subunit (Fig. 3b, right-hand panel). The FLAG-HMK-IL-15 fusion protein 25 does not, however, stimulate the proliferation of parental BAF-BO3 cells that were transfected with a vector lacking IL-2R β chain sequences (Fig. 3b, left-hand panel). Thus, FLAG-HMK-IL-15 is a biologically active 30 growth factor that requires expression of IL-2R β chains upon target cells in order to stimulate cellular proliferation.

- 35 -

present in the supernatants of these cell lysates were separated by 8% SDS-PAGE under reducing conditions. An autoradiogram was developed from the dry gel after exposure at -70°C overnight.

5

Cell staining for flow cytometry

Resting or PHA-activated PBMCs (3×10^5 cells/tube) were washed twice with ice-cold PBS/0.02% sodium azide and incubated in medium containing FLAG-HMK-IL-15 (0.1 μ g/100 μ l) or, as a control, in medium alone. 10 The incubation was carried out on ice for 30 minutes. The cells were then washed with PBS and incubated for 2 sequential 30-minute periods at 4°C with biotin-conjugated anti-FLAG monoclonal antibody (0.5 μ g/100 μ l) and streptavidin-RED670 (0.5 μ g/100 μ l). The cells were 15 then counterstained with CD25-PE, CD14-PE, CD16-PE, CD122-PE, CD4-FITC, or CD8-FITC, as indicated. Addition of IgG1-PE or IgG1-FITC was added to cell populations that had not been incubated with any of the aforementioned reagents in order to identify non-specific 20 binding. Cell surface phenotype was analyzed using FACScan 1 (Becton/Dickinson, CA) and Cell Quest software.

FLAG-HMK-IL-15 binds the IL-15R α subunit

The purified FLAG-HMK-IL-15 fusion protein was tested to determine whether it interacts with cell 25 surface IL-15 receptors. As described above, [32 P]-FLAG-HMK-IL-15 was added to cultures of PBMCs that were activated by a mitogen, PHA. In order to permanently bind interactive proteins to one another, the chemical cross-linker disuccinimidyl suberate (DSS) was added. 30 The cells were washed, lysed, centrifuged, and detergent-soluble proteins were separated by SDS-PAGE. Autoradiography of SDS-PAGE separated proteins revealed a single 75-80 kDa band corresponding to the combined

- 38 -

positive) were noted (Fig. 5B). Interestingly, there are IL-2R β cells that fail to bind IL-15 (Fig. 5B). Therefore, expression of IL-2R β chains is not sufficient for IL-15 binding.

5 Taken together, these data indicate that IL-15 can bind IL-15R α , IL-2R α , IL-2R β cells. A similar conclusion was reached through experimentation that probed the interaction of IL-15 with IL-2R α , β cells transfected with IL-15R α subunit (Anderson et al., J. 10 Biol. Chem. 270:29862, 1995; Giri et al., EMBO J. 14:3654, 1995).

In addition to the requirement for IL-15R α subunit expression, the IL-2R β and IL-2R γ subunits are required to render cells sensitive to IL-15 triggered growth (Fig. 15 3, see also Fig. 5).

Dexamethasone, but not Cyclosporine or Rapamycin
Blocks Mitogen-induced Expression of the IL-15R α
Subunit

The effects of several immunosuppressive drugs on 20 PHA-induced expression of IL-15R α by PBMCs were also studied. The drugs tested were cyclosporine-A (CsA), rapamycin, and dexamethasone. The addition of CsA to PBMCs cultured with PHA resulted in a 20% reduction in the expression of IL-2R α , but IL-15R α was unaffected 25 (Fig. 6). The addition of rapamycin actually resulted in enhanced IL-15R α expression (Fig. 6C). In contrast to the effects of CsA and rapamycin, dexamethasone powerfully blocked the expression of IL-15R α chains on mitogen activated PBMCs (Fig. 6D). In accordance with these 30 data, PHA-activated, dexamethasone-treated cells proliferated poorly in response to IL-15, while the response to IL-2 was not inhibited by dexamethasone (Fig. 7). In this experiment, PMBCs were cultured in the presence of PHA and various immuno-suppressive drugs, and 35 then washed and cultured further in the presence of

- 37 -

Mitogen-activated, but not Resting, PBMCs Express the IL-15R α Subunit

The FLAG-HMK-IL-15 fusion protein, biotinylated anti-FLAG antibody, and streptavidin-RED670 were employed 5 to detect expression of IL-15 binding sites on human PBMCs by cytofluorometric analysis. The PBMCs tested were either freshly isolated or PHA-activated. These cells were washed and incubated with either medium alone or FLAG-HMK-IL-15 followed by anti-FLAG biotinylated Ab 10 and streptavidin-RED670. The stained cells were analyzed by flow cytometry (Fig. 4A). PBMCs that were activated with PHA expressed IL-15R α proteins but resting PBMCs did not. In keeping with the result of the cross-linking experiments described above (Fig. 2), binding of FLAG- 15 HMK-IL-15 to PHA activated PBMCs is blocked by a molar excess of rIL-15 (Fig. 4B), thereby demonstrating the specificity of FLAG-HMK-IL-15 binding for IL-15 binding sites. Both activated CD4 $^{+}$ and CD8 $^{+}$ cells express IL-15 α chains (Fig. 4C). Activation induced IL-15R α chains were 20 also detected on CD14 $^{+}$ (monocyte/macrophage) cells and CD16 $^{+}$ (natural killer) cells.

IL-2R α and IL-2R β Subunits Are Not Required for IL-15 Binding

FACS analysis of PHA-activated PBMCs stained with 25 FLAG-HMK-IL-15 proteins and anti-CD25 Mab, against the IL-2R α subunit, reveals cell populations expressing both IL-15R α and IL-2R α subunits, as well as cell populations that express either subunit, but not both (Fig. 5A). There are IL-2R α cells that do not bind FLAG-HMK-IL-15 30 (Fig. 5A). Almost all PBMCs that were stimulated with PHA for only one day express either IL-15R α or IL-2R β chains, but not both proteins (Fig. 5B). In contrast, 3 days following PHA stimulation, a far larger population of IL-15R α , IL-2R β cells (double positive) and a far 35 smaller population of IL-15R α , IL-2R β cells (single

- 40 -

results, in conjunction with the data showing CsA-independent induction of IL-15 gene expression, suggest that CsA may not block IL-15 triggered immune responses. On the contrary, efficiently blocking the 5 induction of the IL-15R α subunit with dexamethasone may be very helpful in preventing IL-15 induced cellular proliferation.

In summary, the experiments presented above have demonstrated that: (i) IL-15R α subunits are rapidly 10 expressed by activated macrophages, T cells, and NK cells, and (ii) induction of the IL-15R α subunit is blocked by dexamethasone but not by CsA or rapamycin. In addition, the experiments have confirmed that the IL-15R α subunit is necessary and sufficient for IL-15 binding and 15 that the FLAG-HMK-IL-15 fusion protein is an extremely useful tool for studying IL-15 receptors.

A COMPARATIVE ANALYSIS OF THE IL-2 AND IL-15 SIGNALLING PATHWAYS

In this series of experiments, the intracellular 20 signalling pathway that is initiated when IL-2 binds to the IL-2R α , - β , - γ receptor complex was compared to the pathway initiated when IL-15 binds to the receptor complex composed of IL-15R α , IL-2R β , and IL-2R γ .

The methods, in addition to those described above, 25 required to perform these experiments follow.

Cell Culture, Cell Lysis and Protein Immunoblotting

Fresh human PBMCs were isolated as described above and cultured in RPMI-1640 medium supplemented with 30 10% FCS, penicillin, and streptomycin (also as above). To achieve activation by a mitogen, PHA (10 μ g/ml) was added to the culture for 3 days. PHA-stimulated PBMCs were washed 3 times with PBS, cultured for 14 hours in RPMI-1640 medium containing 10% FCS, penicillin, and

- 39 -

medium alone ("none"), PHA, IL-2, or IL-15 for 2 days before a [³H]-TdR pulse. The potent response of PHA-activated, dexamethasone-treated PBMCS to IL-2 proves that the ability of dexamethasone to inhibit the 5 proliferative response to IL-15 is not due to a non-specific toxic effect.

The studies described above used FLAG-HMK-IL-15 and flow cytometry analysis to determine whether resting and mitogen-activated PBMCS express the IL-15R α subunit. 10 The IL-15R α subunit is rapidly expressed by activated but not resting lymphocytes, NK-cells, and macrophages (Fig. 4).

These studies have also shown that the induction of IL-15R α by mitogen is sensitive to an 15 immunosuppressant: PBMCS pre-treated with dexamethasone and stimulated with PHA do not express IL-15R α as vigorously as cells that were not pre-treated with dexamethasone (Fig. 6). Similarly, PBMCS pre-treated with dexamethasone do not proliferate as robustly in 20 response to exogenously added IL-15 (Fig. 7). The cells do respond partially to IL-15. This may reflect that fact that IL-15R α is expressed within 24 hours of dexamethasone withdrawal. CsA, a potent inhibitor of IL-2 and IL-2R α expression (Farrar et al., J. Biol. Chem. 25 264:12562, 1989), is ineffective in preventing the mitogen-induced expression of the IL-15R α chain (Fig. 6). There was a slight reduction in the proliferative response to exogenously added IL-15 among cells pre-treated with CsA for 72 hours (Fig. 7), however this 30 reduction may be due to inhibition of any number of events in the CsA-sensitive, Ca²⁺-dependent pathway of T cell activation. Since the same modest reduction in cell proliferation was observed in response to IL-2, CsA may exert certain long lasting effects by blocking 35 signaling pathway(s) shared by IL-2 and IL-15. These

propagates cells that are equally sensitive to further stimulation with either cytokine. Hence, according to the scheme shown in Fig. 8A, stimulation of mitogen-activated PBMCS with IL-2 or IL-15 does not 5 select for a bulk cell population that then responds only to IL-2 or only to IL-15.

Further experiments were carried out using T cells pre-stimulated with PHA for 3 days followed by stimulation with IL-2 for 2 days. IL-2 stimulated cell 10 proliferation is a protein tyrosine kinase dependent event (Maslinski et al. *J. Biol. Chem.* **267**:15281, 1992); Remillard et al., *J. Biol. Chem.* **266**:14167, 1991). Thus, an inhibitor of tyrosine kinases, genistein, was used to test whether the proliferative signals induced by IL-15 15 are mediated by the same tyrosine kinases as are the proliferative signals induced by IL-2. IL-2 and IL-15 both induce T cell proliferation and both manifest a similar dose-related sensitivity to genistein (Fig. 9).

To rule out the possibility that genistein was 20 functioning as a general toxin, rather than a selective inhibitor of protein kinase-dependent IL-2 or IL-15-induced cell proliferation, a standard trypan blue exclusion assay was performed. Cell viability after 3 days of incubation in the presence of genistein 25 (10 μ g/ml) was 65% \pm 15% of the control (where no genistein was added). Therefore, most of the observed inhibitory effect of genistein is due to inhibition of IL-2 and IL-15 triggered signal transduction.

Tyrosine phosphorylation events are critical for 30 IL-15 stimulated proliferation. Indeed, a direct comparison of the pattern of tyrosine phosphorylation shows that the protein phosphorylation induced by IL-2 is identical to the protein phosphorylation induced by IL-15 (Fig. 10). This indicates that both cytokines stimulate 35 similar, if not identical, protein tyrosine kinases.

- 41 -

streptomycin, then cultured in medium containing recombinant human IL-2 (100 U/ml), recombinant human IL-15 (10 ng/ml) or non-supplemented medium, for 10 minutes at 37°C.

5 After stimulation with interleukins, the cells were washed in cold PBS and lysed in an ice-cold lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mM Na₃VO₄, 50 mM NaF, and 1% NP-40).

10 Lysates were incubated for 15 minutes on ice and then pelleted at 100,000 x g for 30 minutes.

Soluble proteins were separated by SDS-PAGE (10% acrylamide) followed by transfer to a PVDF membrane (Millipore, Bedford, MA). The membrane was then 15 incubated in blocking buffer (25 mM HEPES (pH 7.4), 150 mM NaCl, 0.05% Tween, and 2% BSA) for 2 hours at room temperature, followed by incubation with an anti-phosphotyrosine antibody, RC-20, conjugated to alkaline phosphate (Signal Transduction Labs, Inc. Lexington, KY) 20 for 2 hours at room temperature. Washed blots were developed in the BCIP/NBT phosphatase substrate solution for colorimetric analysis (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, MD).

25 The pattern of Tyrosine Phosphorylated Proteins Induced by IL-2 is the Same as that Induced by IL-15

To select a population of activated, IL-2 responsive T cells, PBMCs were stimulated with PHA for 3 days, washed, and then stimulated with IL-2 or IL-15 30 for an additional 2 days. Cultivation of lymphocytes in IL-2 rich medium leads to propagation of lymphocytes that express a high copy number of the tri-molecular high-affinity IL-2R complex (Maslinski et al., J. Biol. Chem. 267:15281, 1992). Furthermore, 35 cultivation of PHA-treated PBMCs with IL-2 or IL-15

- 44 -

The Immunosuppressant Cyclosporin does not Inhibit Either IL-2 or IL-15 Induced Cellular Proliferation

The effect of a second immunosuppressant, cyclosporine-A (CsA), on IL-15 induced cell proliferation was also examined. Cyclosporine-A is a peptide that forms complexes with cyclophilin; these complexes bind to and inhibit the enzymatic activity of the cellular phosphate calcineurin, resulting, among other effects, in blockade of IL-2 and IL-2R α gene transcription (Sigal et al., *Annu. Rev. Immunol.* 10:519, 1992). T cells expressing the tri-molecular IL-2R complex and stimulated with exogenously added IL-2 are not sensitive to CsA (Sigal et al., *Annu. Rev. Immunol.* 10:519, 1992). It has now been shown that CsA does not inhibit IL-15 or IL-2 triggered cellular proliferation (Fig. 11), again revealing similarities between IL-2 and IL-15 induced cellular proliferation.

The doses of CsA used in these studies (to block PHA-induced IL-2 gene expression) were tested in order to provide evidence that a sufficient amount of CsA was being used. CsA (100 ng/ml) completely blocked PHA-induced IL-2 mRNA expression as judged by the results of RT-PCR and competitive PCR techniques, as described before (Lipman et al., *J. Immunol.* 152:5120, 1994). This result confirms the efficacy of CsA in the culture system used herein. Moreover, it discounts the possibility that IL-15 induces IL-2 gene expression that, in turn, could be responsible for T cell proliferation.

30 The IL-2R β Subunit is Critical for both IL-2 and IL-15 Signal Transduction

The hypothesis that elements of the IL-2R β chain that are required for IL-2 signal transduction are also required for IL-15 signal transduction was tested.

35 Previous studies indicated that BAF-BO3 cells that

- 43 -

IL-2 and IL-15 Stimulated Proliferative Events are Equally Sensitive to Inhibition by Rapamycin

To further characterize the signaling events that are mediated by IL-2 and IL-15, the sensitivity of IL-2 and IL-15 induced T cell proliferation to rapamycin, which inhibits IL-2R signal transduction. Rapamycin, a macrolide with potent immunosuppressive activity (Sigal et al., *Annu. Rev. Immunol.* 10:519, 1992; Sehgal et al., *ASHI Quarterly* 15:8, 1991), inhibits IL-2 induced signal transduction prior to or at the level of activation of p70 S6 kinase and cyclin E dependent cdk2 kinase (Price et al., *Science* 257:973, 1992; Bierer et al., *Proc. Natl. Acad. Sci. USA* 87:9231, 1990; Calvo et al., *Proc. Natl. Acad. Sci. USA* 89:7571, 1992; and Chung et al., *Cell* 69:1227, 1992). PHA-stimulated, IL-2-activated cells were washed prior to incubation with rapamycin and cultured with IL-2 or IL-15. Again, the similarity of IL-2 and IL-15 induced T cell proliferative events was evident: the proliferative events stimulated by these two interleukins were equally dose-sensitive to inhibition by rapamycin (Fig. 10). Just as in studies involving genistein (see above), the possibility that rapamycin was toxic to lymphocytes was ruled out by performing standard trypan blue exclusion assays (*Current Protocols in Immunology*, Vol. 3, ed: Coico, R. New York: John Wiley & Sons, 1995). Cell viability after three days of incubation with rapamycin (10 ng/ml) was $8.58 \pm 10\%$ of the control, where no drug was added. Therefore, the observed inhibition of cellular proliferation by rapamycin is due to an inhibition of IL-2 and IL-15 induced signal transduction.

- 46 -

relatively early events in the course of IL-2 and IL-15 intracellular signal transduction, the terminal phase of IL-2 and IL-15 signal transduction are also likely to be quite similar insofar as activation of target cells with 5 IL-2 or IL-15 gives rise to expression of the same DNA binding proteins (Giri et al., *EMBO J.* 13:2822, 1994). These results also suggest that the function of the IL-15R α and IL-2R α chain are similar, i.e. that they are most important for cytokine binding affinity and have a 10 negligible role in signal transduction.

Decreasing the viability of activated T cells or blocking the signal transduction pathways activated by IL-2 and IL-15 provides a way to decrease the production of lymphokines and mitogens that contribute to 15 accelerated atherosclerosis, allograft rejection, certain leukemias and other immune-mediated pathologies. When activated, T cells proliferate and express receptors on their cell surface for interleukins. In addition, activated T cells release at least 3 lymphokines: gamma 20 interferon, B cell differentiation factor II, and IL-3. These lymphokines can produce various undesirable events, such as allograft rejection. In contrast, resting T cells and long-term memory T cells do not express lymphokine receptors. This difference in receptor 25 expression provides a means to target activated immune cells without interfering with resting cells. Molecules designed to recognize some subunit of the IL-15R will recognize activated monocytes/macrophages as well as activated T cells and can be used to selectively inhibit 30 or destroy these cells. Derivatives of IL-15 that bind to an IL-15R subunit but that lack IL-15 activity, either because they block the binding and/or uptake of bona fide IL-15, are useful in the method of the invention. The mutant IL-15 molecule described below provides a working 35 example of such a derivative.

- 45 -

express endogenous IL-2R α and IL-2R γ subunits can be rendered IL-2 dependent upon transfection with cDNA encoding human the IL-2R β protein (Hatakeyama et al., *Science* 244:551, 1989). In contrast, BAF-BO3 cells that 5 express a mutant IL-2R β protein, which lacks a 71 amino acid serine-rich region, do not respond to IL-2 (Hatakeyama et al., *Science* 252:1523, 1991; Hatakeyama et al., *Science* 244:551, 1989; and Fung et al., *J. Immunol.* 147:1253, 1991). Therefore, the ability of IL-15 to 10 induce the proliferation of IL-2R $\alpha\gamma$ BAF-BO3 cells that express either the wild-type IL-2R β subunit or a mutant IL-2R β protein that lacks the serine rich region (S'). While both IL-2 and IL-15 stimulate the proliferation of 15 cells that express the wild-type IL-2R β subunit, neither cytokine is able to stimulate IL-2R $\alpha\gamma$ BAF-BO3 cells that express mutant S' IL-2R β subunit (Fig. 12). These results demonstrate that the serine-rich region of the IL-2R β subunit is critical for both IL-2 and IL-15 triggered signal transduction.

20 The serine-rich region of the IL-2R β subunit binds several tyrosine kineses including Jak-1 and Syk (Minami et al., *Immunity* 2:89, 1995). The same kinases may play a critical role in both IL-2 and IL-15 signal transduction. Since BAF-BO3 cells cannot express IL-2 25 (Anderson et al., *J. Biol. Chem.* 270:29862, 1995), even upon stimulation with IL-15, these results indicate that IL-15 induced cellular proliferation does not require induction of IL-2 gene expression.

The experiments performed in this series (see also 30 Lin et al., *Immunity* 2:331, 1995; Anderson et al., *J. Biol. Chem.* 270:29862, 1995) indicate that IL-2 and IL-15 triggered signal transduction use overlapping, perhaps identical, signaling pathways, and that agents that block IL-2 signaling are highly likely to block IL-35 15 signaling. Although these experiments compared

- 48 -

Similarly, this strategy can be used to incorporate any other amino acid in place of the glutamine residues at positions 149 or 156 or to introduce amino acid substitutions at positions other than 149 and/or 156.

Proliferation of BAF-BO3 Cells in the Presence of IL-15 Related Proteins

In order to study the effect of various IL-15 related proteins, including the mutant polypeptides described above, on the proliferation of BAF-BO3 cells, the following experiment was performed *in vitro*. BAF-BO3 cells were transfected in culture with DNA from either pRcCMV-IL-2R β or pRcCMV-0. These plasmids differ in that pRcCMV-IL-2R β contains an insert encoding the human IL-15 β subunit and pRcCMV-0 does not. Following transfection, the cells were washed and treated with either: (1) unsupplemented medium ("none"), (2) IL-2, (3) IL-15-rich WEHI cell supernatant (WEHI), (4) FLAG-HMK-IL-15, (5) FLAG-HMK-IL-15-Q149D single mutant (149), (6) FLAG-HMK-IL-15 Q156D double mutant (DM), (7) IL-15, or (8) IL-15 + FLAG-HMK-IL-15 Q149D Q156D double mutant (Fig. 15). Cells transfected with pRcCMV-0 DNA, did not proliferate in response to any stimulus except WEHI cell supernatant. In contrast, following transfection with pRcCMV-IL-2R β DNA, IL-2, WEHI cell supernatant, FLAG-HMK-IL-15, FLAG-HMK-IL-15 Q149D single mutant, and IL-15 stimulated cellular proliferation. The IL-2R β expressing cells did not proliferate in response to the double mutant IL-15. The double mutant IL-15 polypeptide may inhibit BAF-BO3 proliferation in a dose-dependent manner: addition of 30 μ l (approximately 50 μ g/ml) of the double mutant IL-15 inhibited proliferation more completely than did addition of 20 μ L of the same concentration of the double mutant IL-15.

- 47 -

A MUTANT IL-15 POLYPEPTIDE THAT FUNCTIONS AS AN ANTAGONIST OF WILD-TYPE IL-15

Genetic Construction of mutant IL-15

The human IL-15 protein bearing a double mutation 5 (Q149D; Q156D) was designed to target the putative sites critical for binding to the IL-2R γ subunit. The polar, but uncharged glutamine residues at positions 149 and 156 (Fig. 12) were mutated into acidic residues of aspartic acid (Fig. 13) utilizing PCR-assisted mutagenesis. A 10 cDNA encoding the double mutant of IL-15 was amplified by PCR utilizing a synthetic sense oligonucleotide [5'-GGATTCAACTGGGTGAATGTAATA-3' (SEQ ID NO:1); EcoRI site (underlined hexamer) plus bases 145-162] and a synthetic antisense oligonucleotide 15 [5'-CGGGATCCTCAAGAAGTGTGATGAAACATGTCGACAATATGTACAAAACTGTC CAAAAAT-3' (SEQ ID NO:3); BamHI site (underlined hexamer) plus bases 438-489; mutated bases are singly underlined]. The template was a plasmid containing cDNA that encodes human FLAG-HMK-IL-15. The amplified fragment was 20 digested with EcoRI/BamHI and cloned into the pAR(DRI)59/60 plasmid digested with EcoRI/BamRI as described (LeClair et al., Proc. Natl. Acad. Sci. USA 89:8145, 1989). The presence of a mutation at residue 156 was confirmed by digestion with SalI; the mutation 25 introduces a new SalI restriction site. In addition, mutations were verified by DNA sequencing, according to standard techniques. The FLAG-HMK-IL-15 (Q149D; Q156D) double mutant protein was produced, purified, and verified by sequencing as described above for the FLAG- 30 HMK-IL-15 wild-type protein.

Using this same strategy, mutants that contain only a single amino acid substitution, either at position 149 or at position 156 were prepared. As described above, these positions (149 and 156) correspond to 35 positions 101 and 108, respectively, in the mature IL-15 polypeptide, which lacks a 48-amino acid signal sequence.

- 50 -

Genetic Construction of Additional Mutant IL-15 Chimeric Polypeptides

In addition to the FLAG-HMK-IL-15 chimera, which provides the mutant IL-15 with an antigenic tag, numerous other polypeptides can be linked to any mutant of IL-15. For example, mutant IL-15 can be linked to serum albumin or to the Fc fragment of the IgG subclass of antibodies, according to the following method.

Genetic Construction of Mutant IL-15/Fc--

10 cDNA for Fc γ 2a can be generated from mRNA extracted from an IgG2a secreting hybridoma using standard techniques with reverse transcriptase (MMLV-RT; Gibco-BRL, Grand Island, NY) and a synthetic oligo-dT (12-18) oligonucleotide (Gibco BRL). The mutant IL-15

15 cDNA can be amplified from a plasmid template by PCR using IL-15 specific synthetic oligonucleotides.

20 The 5' oligonucleotide is designed to insert a unique NotI restriction site 40 nucleotides 5' to the translational start codon, while the 3' oligonucleotide eliminates the termination codon and modifies the C-terminal Ser residue codon usage from AGC to TCG to accommodate the creation of a unique BamHI site at the mutant IL-15/Fc junction. Synthetic oligonucleotides used for the amplification of the Fc γ 2a domain cDNA 25 change the first codon of the hinge from Glu to Asp in order to create a unique BamHI site spanning the first codon of the hinge and introduce a unique XbaI site 3' to the termination codon. The Fc fragment can be modified so that it is non-lytic, i.e., not able to 30 activate the complement system. To make the non-lytic mutant IL-15 construct (mIL-15/Fc--), oligonucleotide site-directed mutagenesis is used to replace the C'1q binding motif Glu318, Lys320, Lys322 with Ala residues. Similarly, Leu235 is replaced with Glu to inactivate the 35 Fc γ R I binding site. Ligation of cytokine and Fc

- 49 -

Proliferation of PHA-Stimulated Human PBMCs in the Presence of IL-15 Related Proteins

Human PBMCs prestimulated with PHA (2 μ g/ml) for 72 hours were washed and cultured in the presence of IL-15 related proteins including: (1) the IL-15 double mutant, FLAG-HMK-IL-15-Q149D-Q156D, (2) the IL-15 single mutant, FLAG-HMK-IL-15-Q149D, or (3) the IL-15 single mutant, FLAG-HMK-IL-15-Q156D (Fig. 16). Medium without an IL-15 related polypeptide served as a control. The proliferative response was then assessed.

FACS Analysis of PHA-Activated Human PBMCs Stained with FLAG-HMK-IL-15-Double Mutants

The ability of the FLAG-HMK-IL-15 double mutant polypeptide to bind PHA-activated human PBMCs was demonstrated as follows. PHA-activated PBMCs were washed and incubated with medium alone, or with the FLAG-HMK-IL-15 double mutant. The cells were then incubated with an anti-FLAG biotinylated antibody and stained with streptavidin conjugated to RED670. The stained cells were analyzed by flow cytometry (Fig. 17).

FACS Analysis of Leukemic Cell Lines Stained with Wild-Type FLAG-HMK-IL-15

In a series of experiments similar to those above, the ability of the wild-type FLAG-HMK-IL-15 polypeptide to bind leukemia cells was shown. The cells treated were obtained from the leukemic cell lines MOLT-14, YT, HuT-102, and from cell lines currently being established at Beth Israel Hospital (Boston, MA), and named 2A and 2B. The cultured cells were washed and incubated with either medium alone or with medium containing the FLAG-HMK-IL-15 wild-type polypeptide (Fig. 18). The cells were then incubated with the biotinylated anti-FLAG antibody and stained with RED670-conjugated streptavidin. The stained cells were analyzed by flow cytometry.

- 52 -

Western blot analysis following SDS-PAGE under reducing (with DTT) and non-reducing (without DTT) conditions can be performed using monoclonal or polyclonal anti-mIL-15 or anti Fc γ primary antibodies to 5 evaluate the size and isotype specificity of the fusion proteins.

Standardization of the Biological Activity of recombinant mutant IL-15 and mIL-15/Fc-- proteins

Using the RT-PCR strategy and 5' NotI sense

10 oligonucleotide primer described above, mutant IL-15 cDNA with an *Xba*I restriction site added 3' to its native termination codon, can be cloned into pRc/CMV. This construct is then transiently expressed in COS cells (available from the American Type Culture Collection).
15 The cells may be transfected by the DEAE dextran method and grown in serum-free UltraCulture media (BioWhittaker Inc.). Day 5 culture supernatant is sterile filtered and stored at -20°C for use as a source of recombinant mutant IL-15 protein (rmIL-15).
20 Mutant IL-15/Fc-- and rmIL-15 mutant protein concentrations can be determined by ELISA as well as by bioassay, as described (Thompson-Snipes et al., *J. Exp. Med.* 173:507 (1991)).

25 The functional activity of mutant IL-15/Fc-- can be assessed by a standard T cell proliferation assay, as described above.

Determination of mIL-15/Fc-- or mIL-15/Fc++ Circulating Half-life

Serum concentration of mIL-15/Fc or mIL-15/Fc++ fusion proteins can be determined over time following a 30 single intravenous injection of the fusion protein. Serial 100 μ l blood samples can be obtained by standard measures at intervals of 0.1, 6, 24, 48, 72, and 96 hours after administration of mutant IL-15/Fc-- protein.

- 51 -

components in the correct translational reading frame at the unique *Bam*HI site yields a 1,236 basepair open reading frame encoding a single 411 amino acid polypeptide (including the 18 amino acid IL-15 signal peptide) with a total of 13 cysteine residues. The mature secreted homodimeric IL-15/Fc⁺⁺ is predicted to have a total of up to eight intramolecular and three inter-heavy chain disulfide linkages and a molecular weight of approximately 85 kDa, exclusive of 10² glycosylation.

Section 3. Expression and Purification of mIL-15 Fc Fusion Proteins

Proper genetic construction of both mIL-15/Fc⁺⁺, which carries the wild-type Fc γ 2a sequence, and mIL-15/Fc^{-/-} can be confirmed by DNA sequence analysis following cloning of the fusion genes as *Not*I-*Xba*I cassettes into the eukaryotic expression plasmid pRc/CMV (Invitrogen, San Diego, CA). This plasmid carries a CMV promoter/enhancer, a bovine growth hormone 20 polyadenylation signal and a neomycin resistance gene for selection with G418. Plasmids carrying the mIL-15/Fc⁺⁺ or mIL-15/Fc^{-/-} fusion genes are transfected into Chinese hamster ovary cells (CHO-K1, available from the American Type Culture Collection) by electroporation (1.5 kV/3 25 μ F/0.4 cm/PBS) and selected in serum-free Ultra-CHO media (BioWhittaker Inc., Walkerville, MD) containing 1.5 mg/ml of G418 (Geneticin, Gibco BRL). After subcloning, clones that produce high levels of the fusion protein are selected by screening supernatants from IL-15 by ELISA 30 (PharMingen, San Diego, CA). mIL-15/Fc fusion proteins are purified from culture supernatants by protein A sepharose affinity chromatography followed by dialysis against PBS and 0.22 μ m filter sterilization. Purified proteins can be stored at -20°C before use.

WO 97/41232

- 54 -

5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGGGATCCTC AAGAAGTGT 26

(2) INFORMATION FOR SEQ ID NO:3:

10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 60 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

15 CGGGATCCTC AAGAAGTGT GATGAACATG TCGACAATAT GTACAAAAT GTCCAAAAAT 60

(2) INFORMATION FOR SEQ ID NO:4:

20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asp Tyr Lys Asp Asp Asp Lys
 1 5

25 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 489 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30 (ix) FEATURE:
 (A) NAME/KEY: Coding Sequence
 (B) LOCATION: 1...486
 (D) OTHER INFORMATION:

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG AGA ATT TCG AAA CCA CAT TTG AGA AGT ATT TCC ATC CAG TGC TAC 48
 Met Arg Ile Ser Lys Pro His Leu Arg Ser Ile Ser Ile Gln Cys Tyr
 15 5 10

40 TTG TGT TTA CTT CTA AAC AGT CAT TTT CTA ACT GAA GCT GGC ATT CAT 96
 Leu Cys Leu Leu Leu Asn Ser His Phe Leu Thr Glu Ala Gly Ile His
 20 25 30

GTC TTC ATT TTG GGC TGT TTC AGT GCA GGG CTT CCT AAA ACA GAA GCC 144
 Val Phe Ile Leu Gly Cys Phe Ser Ala Gly Leu Pro Lys Thr Glu Ala
 45 40

- 53 -

Measurements employ an ELISA with a mIL-15 mAb as the capture antibody and horseradish peroxidase conjugated to an Fc"2a mAb as the detection antibody, thus assuring this assay is specific for only the mutant IL-15/Fc--.

5

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: BETH-ISRAEL HOSPITAL ASSOCIATION

(ii) TITLE OF THE INVENTION: ANTAGONISTS OF INTERLEUKIN-15

(iii) NUMBER OF SEQUENCES: 7

10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Fish & Richardson, P.C.

(B) STREET: 225 Franklin Street

15

(C) CITY: Boston

(D) STATE: MA

(E) COUNTRY: US

(F) ZIP: 02110-2804

20

(v) COMPUTER READABLE FORM

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: Windows95

(D) SOFTWARE: FastSEQ for Windows Version 2.0

25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT/US97/06931

(B) FILING DATE: 25-APR-1997

(C) CLASSIFICATION:

30

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 60/016,634

(B) FILING DATE: 26-APR-1996

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Freeman, John W.

(B) REGISTRATION NUMBER: 29,066

(C) REFERENCE/DOCKET NUMBER: 01948/039WO1

35

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-542-5070

(B) TELEFAX: 617-542-8906

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(x) SEQUENCE DESCRIPTION: SEQ. ID. NO:1:

GGAATTCAAC TGGGTGAATG TAATA 25

AGG ADA 3AA TCC TTD AGG AGG TCA 3AA TCA

ATG AGT AGG TTT AGG AGG TCA 3AA TCA

(2) INFORMATION FOR SEQ ID NO:2:

WO 97/41232

- 56 -

Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val Glu
 100 105 110
 Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn Val
 115 120 125
 5 Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Lys Asn Ile
 130 135 140
 Lys Glu Phe Leu Gln Ser Phe Val His Ile Val Gln Met Phe Ile Asn
 145 150 155 160
 Thr Ser

10 (2) INFORMATION FOR SEQ ID NO:7:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 489 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 15 (D) TOPOLOGY: linear
 (ix) FEATURE:
 (A) NAME/KEY: Coding Sequence
 (B) LOCATION: 1...486
 (D) OTHER INFORMATION:
 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
 ATG AGA ATT TCG AAA CCA CAT TTG AGA AGT ATT TCC ATC CAG TGC TAC 48
 Met Arg Ile Ser Lys Pro His Leu Arg Ser Ile Ser Ile Gln Cys Tyr
 1 5 10 15
 TTG TGT TTA CTT CTA AAC AGT CAT TTT CTA ACT GAA GCT GGC ATT CAT 96
 25 Leu Cys Leu Leu Asn Ser His Phe Leu Thr Glu Ala Gly Ile His
 20 25 30
 GTC TTC ATT TTG GGC TGT TTC AGT GCA GGG CTT CCT AAA ACA GAA GCC 144
 Val Phe Ile Leu Gly Cys Phe Ser Ala Gly Leu Pro Lys Thr Glu Ala
 35 40 45
 30 AAC TGG GTG AAT GTA ATA AGT GAT TTG AAAAATT GAA GAT CTT ATT 192
 Asn Trp Val Asn Val Ile Ser Asp Leu Lys Lys Ile Glu Asp Leu Ile
 50 55 60
 CAA TCT ATG CAT ATT GAT GCT ACT TTA TAT ACG GAA AGT GAT GTT CAC 240
 Gln Ser Met His Ile Asp Ala Thr Leu Tyr Thr Glu Ser Asp Val His
 65 70 80
 CCC AGT TGC AAA GTA ACA GCA ATG AAG TGC TTT CTC TTG GAG TTA CAA 288
 Pro Ser Cys Lys Val Thr Ala Met Lys Cys Phe Leu Glu Leu Gln
 85 90 95
 40 GTT ATT TCA CTT GAG TCC GGA GAT GCA AGT ATT CAT GAT ACA GTA GAA 336
 Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val Glu
 100 105 110
 AAT CTG ATC ATC CTA GCA AAC AAC AGT TTG TCT TCT AAC GGG AAT GTA 384
 Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn Val
 115 120 125
 45 ACA GAA TCT GGA TGC AAA GAA TGT GAG GAA CTG GAG GAA AAA AAT ATT 432
 Thr Glu Ser Gly Cys Lys Glu Cys Glu Leu Glu Lys Asn Ile

- 55 -

AAC TGG GTG AAT GTA ATA AGT GAT TTG AAA AAA ATT GAA GAT CTT ATT 192
 Asn Trp Val Asn Val Ile Ser Asp Leu Lys Lys Ile Glu Asp Leu Ile
 50 55 60
 CAA TCT ATG CAT ATT GAT GCT ACT TTA TAT ACG GAA AGT GAT GTT CAC 240
 Gln Ser Met His Ile Asp Ala Thr Leu Tyr Thr Glu Ser Asp Val His
 65 70 75 80
 CCC AGT TGC AAA GTA ACA GCA ATG AAG TGC TTT CTC TTG GAG TTA CAA 288
 Pro Ser Cys Lys Val Thr Ala Met Lys Cys Phe Leu Leu Glu Leu Gln
 85 90 95
 10 GTT ATT TCA CTT GAG TCC GGA GAT GCA AGT ATT CAT GAT ACA GTA GAA 336
 Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val Glu
 100 105 110
 AAT CTG ATC ATC CTA GCA AAC AAC AGT TTG TCT TCT AAC GGG AAT GTA 384
 Asn Leu Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn Val
 115 120 125
 ACA GAA TCT GGA TGC AAA GAA TGT GAG GAA CTG GAG GAA AAA AAT ATT 432
 Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Glu Lys Asn Ile
 130 135 140
 20 AAA GAA TTT TTG CAG AGT TTT GTA CAT ATT GTC CAA ATG TTC ATC AAC 480
 Lys Glu Phe Leu Gln Ser Phe Val His Ile Val Gln Met Phe Ile Asn
 145 150 155 160
 ACT TCT TGA 489
 Thr Ser Val Ile Val Gln Met Phe Ile Asn
 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395 400 405 410 415 420 425 430 435 440 445 450 455 460 465 470 475 480 485 490 495 500 505 510 515 520 525 530 535 540 545 550 555 560 565 570 575 580 585 590 595 600 605 610 615 620 625 630 635 640 645 650 655 660 665 670 675 680 685 690 695 700 705 710 715 720 725 730 735 740 745 750 755 760 765 770 775 780 785 790 795 800 805 810 815 820 825 830 835 840 845 850 855 860 865 870 875 880 885 890 895 900 905 910 915 920 925 930 935 940 945 950 955 960 965 970 975 980 985 990 995 1000 1005 1010 1015 1020 1025 1030 1035 1040 1045 1050 1055 1060 1065 1070 1075 1080 1085 1090 1095 1100 1105 1110 1115 1120 1125 1130 1135 1140 1145 1150 1155 1160 1165 1170 1175 1180 1185 1190 1195 1200 1205 1210 1215 1220 1225 1230 1235 1240 1245 1250 1255 1260 1265 1270 1275 1280 1285 1290 1295 1300 1305 1310 1315 1320 1325 1330 1335 1340 1345 1350 1355 1360 1365 1370 1375 1380 1385 1390 1395 1400 1405 1410 1415 1420 1425 1430 1435 1440 1445 1450 1455 1460 1465 1470 1475 1480 1485 1490 1495 1500 1505 1510 1515 1520 1525 1530 1535 1540 1545 1550 1555 1560 1565 1570 1575 1580 1585 1590 1595 1600 1605 1610 1615 1620 1625 1630 1635 1640 1645 1650 1655 1660 1665 1670 1675 1680 1685 1690 1695 1700 1705 1710 1715 1720 1725 1730 1735 1740 1745 1750 1755 1760 1765 1770 1775 1780 1785 1790 1795 1800 1805 1810 1815 1820 1825 1830 1835 1840 1845 1850 1855 1860 1865 1870 1875 1880 1885 1890 1895 1900 1905 1910 1915 1920 1925 1930 1935 1940 1945 1950 1955 1960 1965 1970 1975 1980 1985 1990 1995 2000 2005 2010 2015 2020 2025 2030 2035 2040 2045 2050 2055 2060 2065 2070 2075 2080 2085 2090 2095 2100 2105 2110 2115 2120 2125 2130 2135 2140 2145 2150 2155 2160 2165 2170 2175 2180 2185 2190 2195 2200 2205 2210 2215 2220 2225 2230 2235 2240 2245 2250 2255 2260 2265 2270 2275 2280 2285 2290 2295 2300 2305 2310 2315 2320 2325 2330 2335 2340 2345 2350 2355 2360 2365 2370 2375 2380 2385 2390 2395 2400 2405 2410 2415 2420 2425 2430 2435 2440 2445 2450 2455 2460 2465 2470 2475 2480 2485 2490 2495 2500 2505 2510 2515 2520 2525 2530 2535 2540 2545 2550 2555 2560 2565 2570 2575 2580 2585 2590 2595 2600 2605 2610 2615 2620 2625 2630 2635 2640 2645 2650 2655 2660 2665 2670 2675 2680 2685 2690 2695 2700 2705 2710 2715 2720 2725 2730 2735 2740 2745 2750 2755 2760 2765 2770 2775 2780 2785 2790 2795 2800 2805 2810 2815 2820 2825 2830 2835 2840 2845 2850 2855 2860 2865 2870 2875 2880 2885 2890 2895 2900 2905 2910 2915 2920 2925 2930 2935 2940 2945 2950 2955 2960 2965 2970 2975 2980 2985 2990 2995 3000 3005 3010 3015 3020 3025 3030 3035 3040 3045 3050 3055 3060 3065 3070 3075 3080 3085 3090 3095 3100 3105 3110 3115 3120 3125 3130 3135 3140 3145 3150 3155 3160 3165 3170 3175 3180 3185 3190 3195 3200 3205 3210 3215 3220 3225 3230 3235 3240 3245 3250 3255 3260 3265 3270 3275 3280 3285 3290 3295 3300 3305 3310 3315 3320 3325 3330 3335 3340 3345 3350 3355 3360 3365 3370 3375 3380 3385 3390 3395 3400 3405 3410 3415 3420 3425 3430 3435 3440 3445 3450 3455 3460 3465 3470 3475 3480 3485 3490 3495 3500 3505 3510 3515 3520 3525 3530 3535 3540 3545 3550 3555 3560 3565 3570 3575 3580 3585 3590 3595 3600 3605 3610 3615 3620 3625 3630 3635 3640 3645 3650 3655 3660 3665 3670 3675 3680 3685 3690 3695 3700 3705 3710 3715 3720 3725 3730 3735 3740 3745 3750 3755 3760 3765 3770 3775 3780 3785 3790 3795 3800 3805 3810 3815 3820 3825 3830 3835 3840 3845 3850 3855 3860 3865 3870 3875 3880 3885 3890 3895 3900 3905 3910 3915 3920 3925 3930 3935 3940 3945 3950 3955 3960 3965 3970 3975 3980 3985 3990 3995 4000 4005 4010 4015 4020 4025 4030 4035 4040 4045 4050 4055 4060 4065 4070 4075 4080 4085 4090 4095 4100 4105 4110 4115 4120 4125 4130 4135 4140 4145 4150 4155 4160 4165 4170 4175 4180 4185 4190 4195 4200 4205 4210 4215 4220 4225 4230 4235 4240 4245 4250 4255 4260 4265 4270 4275 4280 4285 4290 4295 4300 4305 4310 4315 4320 4325 4330 4335 4340 4345 4350 4355 4360 4365 4370 4375 4380 4385 4390 4395 4400 4405 4410 4415 4420 4425 4430 4435 4440 4445 4450 4455 4460 4465 4470 4475 4480 4485 4490 4495 4500 4505 4510 4515 4520 4525 4530 4535 4540 4545 4550 4555 4560 4565 4570 4575 4580 4585 4590 4595 4600 4605 4610 4615 4620 4625 4630 4635 4640 4645 4650 4655 4660 4665 4670 4675 4680 4685 4690 4695 4700 4705 4710 4715 4720 4725 4730 4735 4740 4745 4750 4755 4760 4765 4770 4775 4780 4785 4790 4795 4800 4805 4810 4815 4820 4825 4830 4835 4840 4845 4850 4855 4860 4865 4870 4875 4880 4885 4890 4895 4900 4905 4910 4915 4920 4925 4930 4935 4940 4945 4950 4955 4960 4965 4970 4975 4980 4985 4990 4995 5000 5005 5010 5015 5020 5025 5030 5035 5040 5045 5050 5055 5060 5065 5070 5075 5080 5085 5090 5095 5100 5105 5110 5115 5120 5125 5130 5135 5140 5145 5150 5155 5160 5165 5170 5175 5180 5185 5190 5195 5200 5205 5210 5215 5220 5225 5230 5235 5240 5245 5250 5255 5260 5265 5270 5275 5280 5285 5290 5295 5300 5305 5310 5315 5320 5325 5330 5335 5340 5345 5350 5355 5360 5365 5370 5375 5380 5385 5390 5395 5400 5405 5410 5415 5420 5425 5430 5435 5440 5445 5450 5455 5460 5465 5470 5475 5480 5485 5490 5495 5500 5505 5510 5515 5520 5525 5530 5535 5540 5545 5550 5555 5560 5565 5570 5575 5580 5585 5590 5595 5600 5605 5610 5615 5620 5625 5630 5635 5640 5645 5650 5655 5660 5665 5670 5675 5680 5685 5690 5695 5700 5705 5710 5715 5720 5725 5730 5735 5740 5745 5750 5755 5760 5765 5770 5775 5780 5785 5790 5795 5800 5805 5810 5815 5820 5825 5830 5835 5840 5845 5850 5855 5860 5865 5870 5875 5880 5885 5890 5895 5900 5905 5910 5915 5920 5925 5930 5935 5940 5945 5950 5955 5960 5965 5970 5975 5980 5985 5990 5995 6000 6005 6010 6015 6020 6025 6030 6035 6040 6045 6050 6055 6060 6065 6070 6075 6080 6085 6090 6095 6100 6105 6110 6115 6120 6125 6130 6135 6140 6145 6150 6155 6160 6165 6170 6175 6180 6185 6190 6195 6200 6205 6210 6215 6220 6225 6230 6235 6240 6245 6250 6255 6260 6265 6270 6275 6280 6285 6290 6295 6300 6305 6310 6315 6320 6325 6330 6335 6340 6345 6350 6355 6360 6365 6370 6375 6380 6385 6390 6395 6400 6405 6410 6415 6420 6425 6430 6435 6440 6445 6450 6455 6460 6465 6470 6475 6480 6485 6490 6495 6500 6505 6510 6515 6520 6525 6530 6535 6540 6545 6550 6555 6560 6565 6570 6575 6580 6585 6590 6595 6600 6605 6610 6615 6620 6625 6630 6635 6640 6645 6650 6655 6660 6665 6670 6675 6680 6685 6690 6695 6700 6705 6710 6715 6720 6725 6730 6735 6740 6745 6750 6755 6760 6765 6770 6775 6780 6785 6790 6795 6800 6805 6810 6815 6820 6825 6830 6835 6840 6845 6850 6855 6860 6865 6870 6875 6880 6885 6890 6895 6900 6905 6910 6915 6920 6925 6930 6935 6940 6945 6950 6955 6960 6965 6970 6975 6980 6985 6990 6995 7000 7005 7010 7015 7020 7025 7030 7035 7040 7045 7050 7055 7060 7065 7070 7075 7080 7085 7090 7095 7100 7105 7110 7115 7120 7125 7130 7135 7140 7145 7150 7155 7160 7165 7170 7175 7180 7185 7190 7195 7200 7205 7210 7215 7220 7225 7230 7235 7240 7245 7250 7255 7260 7265 7270 7275 7280 7285 7290 7295 7300 7305 7310 7315 7320 7325 7330 7335 7340 7345 7350 7355 7360 7365 7370 7375 7380 7385 7390 739

- 58 -

1. A substantially pure mutant IL-15 polypeptide, the polypeptide being:

(a) at least 90% identical to wild-type IL-15, and
(b) capable of inhibiting at least one of the
5 cellular events that normally occurs when wild-type IL-15
specifically binds to a cell surface receptor.

2. The mutant polypeptide of claim 1, wherein the mutant polypeptide competes with wild-type IL-15 for binding to a cell surface receptor.

10 3. The mutant polypeptide of claim 1, wherein the mutant polypeptide binds to a cell surface receptor with at least as high an affinity as wild-type IL-15 binds the same cell surface receptor.

15 4. The mutant polypeptide of claim 1, wherein the mutant polypeptide binds the cell surface receptor with a Kd of greater than 10^{-9} M.

5. The mutant polypeptide of claim 1, wherein at least one mutation is within the domain that binds IL-2R γ .

20 6. The mutant polypeptide of claim 5, wherein the mutation is a deletion, insertion, or substitution of an amino acid residue.

7. The mutant polypeptide of claim 6, wherein the mutation is at position 156 of SEQ ID NO:6.

25 8. The mutant polypeptide of claim 7, further comprising a mutation at position 149 of SEQ ID NO:6.

- 57 -

130

135

140

AAA GAA TTT TTG GAC AGT TTT GTA CAT ATT GTC GAC ATG TTC ATC AAC 480
 Lys Glu Phe Leu Asp Ser Phe Val His Ile Val Asp Met Phe Ile Asn
 145 150 155 160

5 ACT TCT TGA 489
 Thr Ser

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 162 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

15 Met Arg Ile Ser Lys Pro His Leu Arg Ser Ile Ser Ile Gln Cys Tyr
 1 5 10 15
 Leu Cys Leu Leu Leu Asn Ser His Phe Leu Thr Glu Ala Gly Ile His
 20 25 30
 Val Phe Ile Leu Gly Cys Phe Ser Ala Gly Leu Pro Lys Thr Glu Ala
 20 35 40 45
 Asn Trp Val Asn Val Ile Ser Asp Leu Lys Lys Ile Glu Asp Leu Ile
 50 55 60
 Gln Ser Met His Ile Asp Ala Thr Leu Tyr Thr Glu Ser Asp Val His
 65 70 75 80
 25 Pro Ser Cys Lys Val Thr Ala Met Lys Cys Phe Leu Leu Glu Leu Gln
 85 90 95
 Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val Glu
 100 105 110
 Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn Val
 115 120 125
 Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Lys Asn Ile
 130 135 140
 Lys Glu Phe Leu Asp Ser Phe Val His Ile Val Asp Met Phe Ile Asn
 145 150 155 160
 35 Thr Ser

What is claimed is:

- 60 -

18. An isolated nucleic acid molecule encoding the polypeptide of claim 1.

19. A vector comprising the nucleic acid molecule of claim 18.

5 20. The vector of claim 19, wherein the vector is an expression vector.

21. A biological cell comprising the expression vector of claim 20.

22. A method of suppressing the immune response in a patient, the method comprising administering to the patient the mutant IL-15 polypeptide of claim 1.

23. The method of claim 22, wherein the patient has an autoimmune disease or is at risk of developing an autoimmune disease.

15 24. The method of claim 23, wherein the autoimmune disease is a rheumatic disease selected from the group consisting of systemic lupus erythematosus, Sjögren's syndrome, scleroderma, mixed connective tissue disease, dermatomyositis, polymyositis, Reiter's syndrome, and Behcet's disease.

25. The method of claim 23, wherein the autoimmune disease is rheumatic arthritis.

26. The method of claim 23, wherein the autoimmune disease is type I diabetes.

- 59 -

9. The mutant polypeptide of claim 7, wherein the mutation at position 156 of SEQ ID NO:6 is a substitution of aspartate for glutamine.

10. The mutant polypeptide of claim 8, wherein 5 the mutation at position 149 of SEQ ID NO:6 is a substitution of aspartate for glutamine.

11. The mutant polypeptide of claim 1, wherein the polypeptide is part of a chimeric molecule that comprises a heterologous polypeptide.

12. The mutant polypeptide of claim 11, wherein the heterologous polypeptide is the Fc region of IgG.

13. The polypeptide of claim 12, wherein the Fc region of IgG increases the circulating half-life of the mutant polypeptide.

14. The mutant polypeptide of claim 12, wherein the Fc region of IgG is capable of activating the complement lysis system and Fc receptor bearing phagocytes.

15. The mutant polypeptide of claim 12, wherein 20 the Fc region of IgG is incapable of activating the complement lysis system.

16. The mutant polypeptide of claim 1, further comprising an antigenic tag.

17. The mutant polypeptide of claim 16, wherein 25 the antigenic tag is a FLAG sequence comprising Asp-Tyr-Lys-Asp-Asp-Lys (SEQ ID NO:4).

- 62 -

comprising exposing the cell to the mutant IL-15 polypeptide of claim 1.

36. The method of claim 35, wherein the cell is malignant.

5 37. A method of diagnosing a patient as having a disease or condition that could be treated with a mutant IL-15 polypeptide, the method comprising determining whether a biological sample obtained from the patient contains cells that are bound by a polypeptide comprising 10 IL-15 and an antigenic tag, the occurrence of binding indicating that the cells can be bound by mutant IL-15 polypeptide *in vivo* and thereby inhibited from proliferating in response to wild-type IL-15 *in vivo*.

- 61 -

27. The method of claim 23, wherein the autoimmune disease is an autoimmune disease of the thyroid selected from the group consisting of Hashimoto's thyroiditis and Graves' Disease.

5 28. The method of claim 23, wherein the autoimmune disease is an autoimmune disease of the central nervous system selected from the group consisting of multiple sclerosis, myasthenia gravis, and encephalomyelitis.

10 29. The method of claim 23, wherein the autoimmune disease is a variety of pemphigus selected from the group consisting of pemphigus vulgaris, pemphigus vegetans, pemphigus foliaceus, Senechal-Usher syndrome, and Brazilian pemphigus.

15 30. The method of claim 23, wherein the autoimmune disease is psoriasis.

31. The method of claim 23, wherein the autoimmune disease is inflammatory bowel disease.

20 32. The method of claim 22, wherein the patient has acquired immune deficiency syndrome (AIDS).

33. The method of claim 22, wherein the patient has received a transplant of a biological organ, tissue, or cell.

25 34. The method of claim 22, wherein the patient has received a vascular injury.

35. A method of reducing the viability of a cell that expresses a receptor for IL-15, the method

2/18

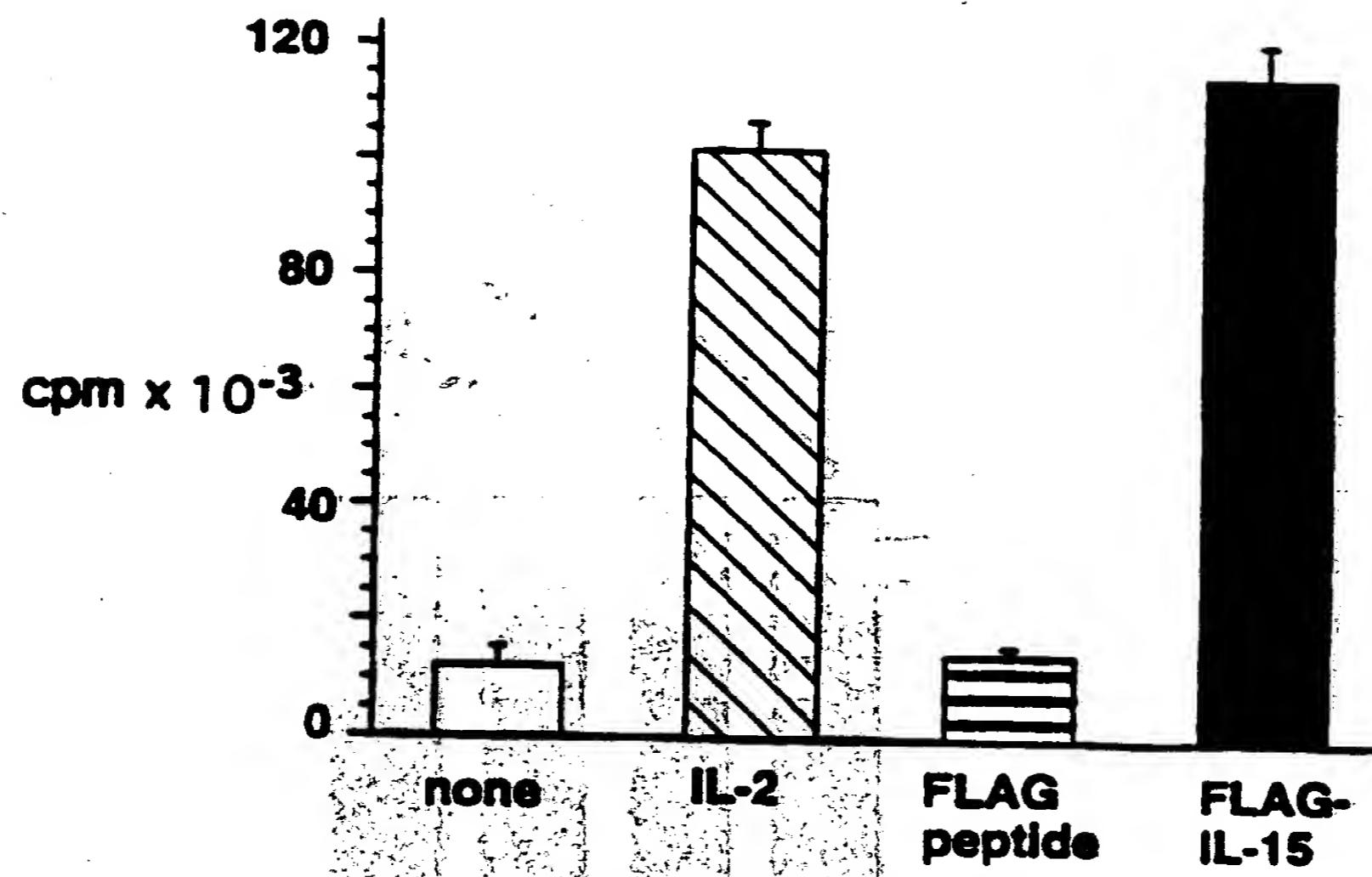


FIG. 3A

BAF-BO3

BAF-BO3-IL-2R β ⁺

- none
- IL-3
- ▨ IL-2
- FLAG-IL-15

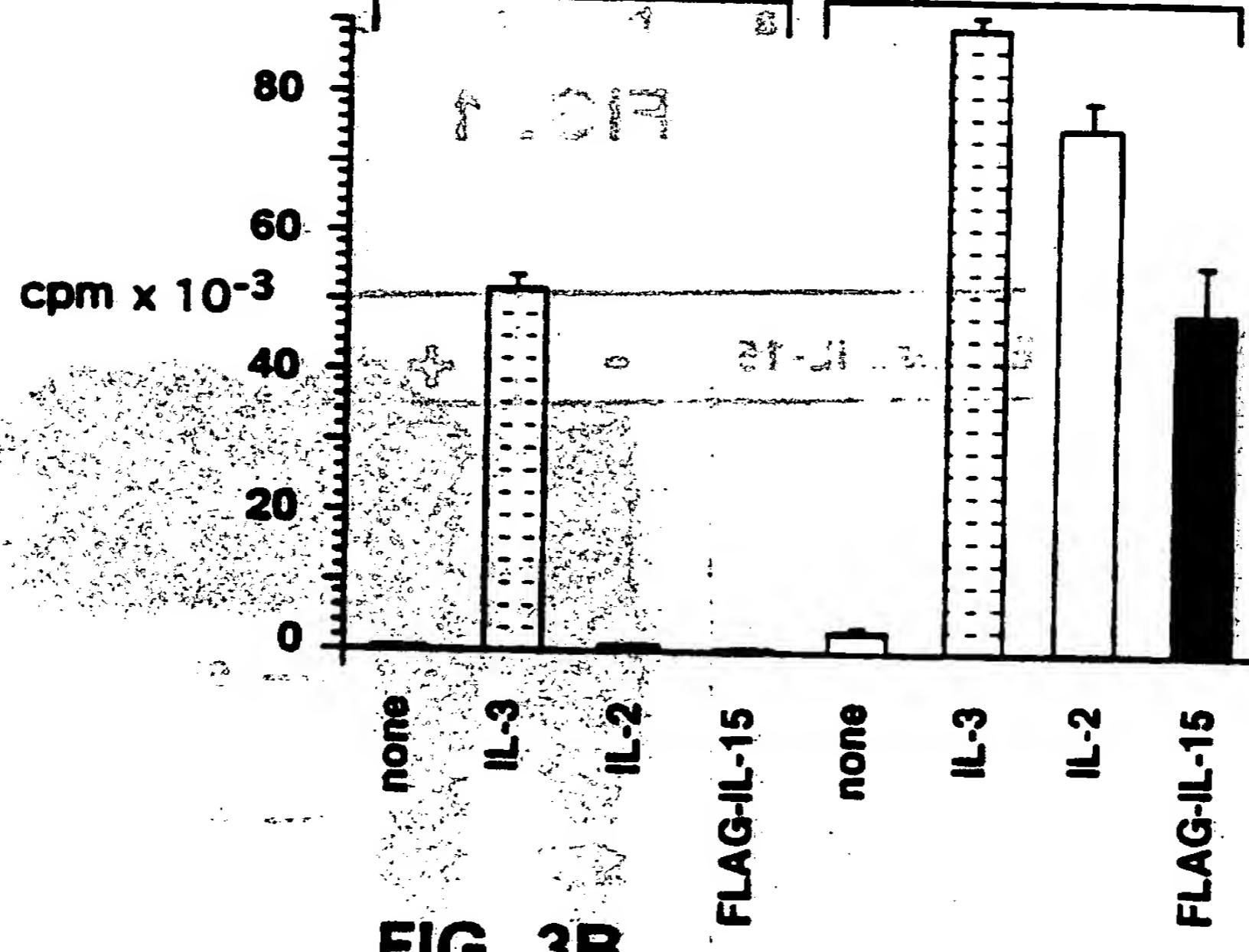


FIG. 3B

SUBSTITUTE SHEET (RULE 26)

1/18

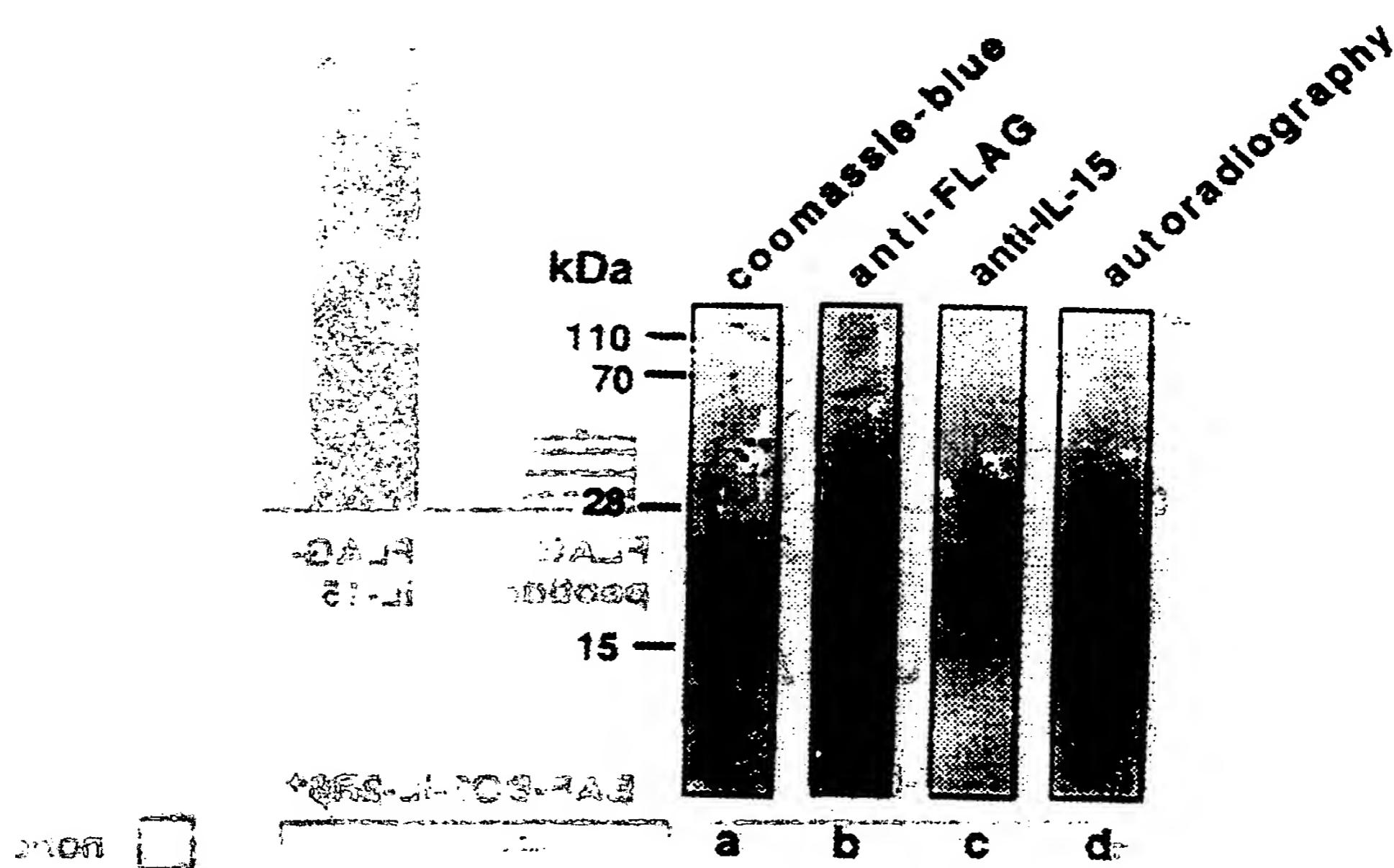


FIG. 1

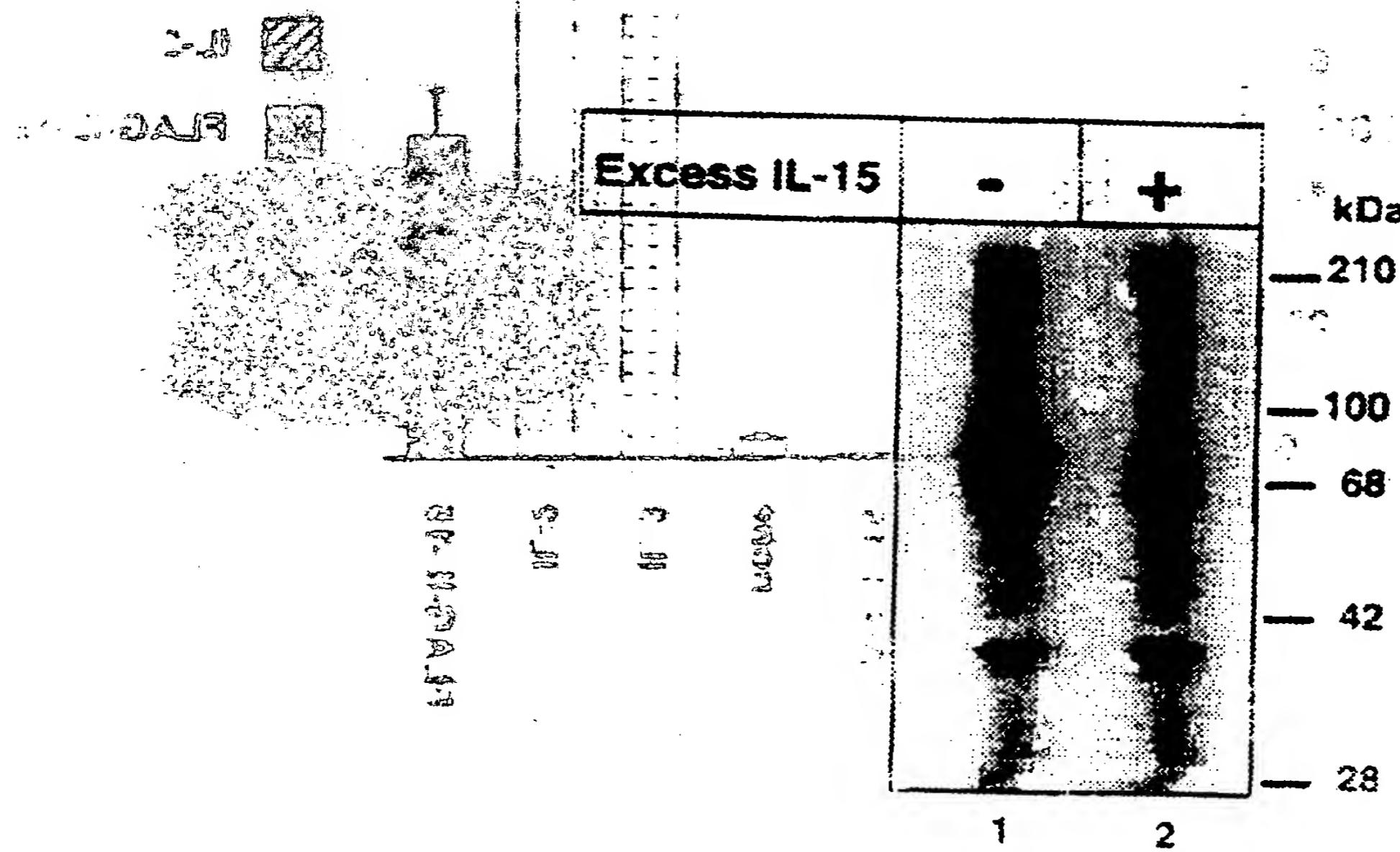


FIG. 2

SUBSTITUTE SHEET (RULE 26)

4/18

FIG. 4C

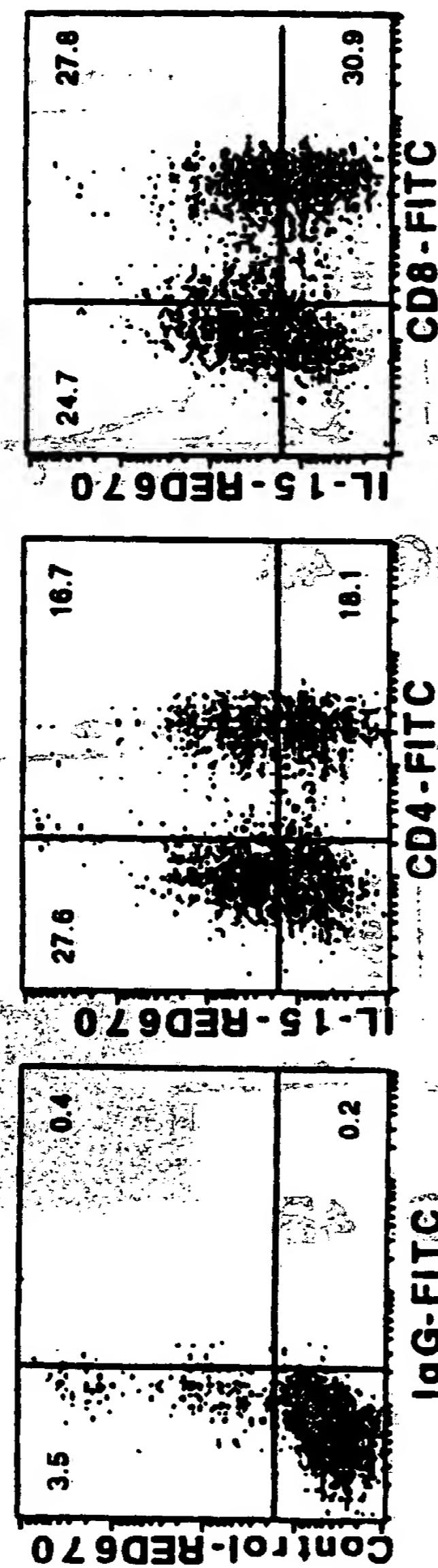
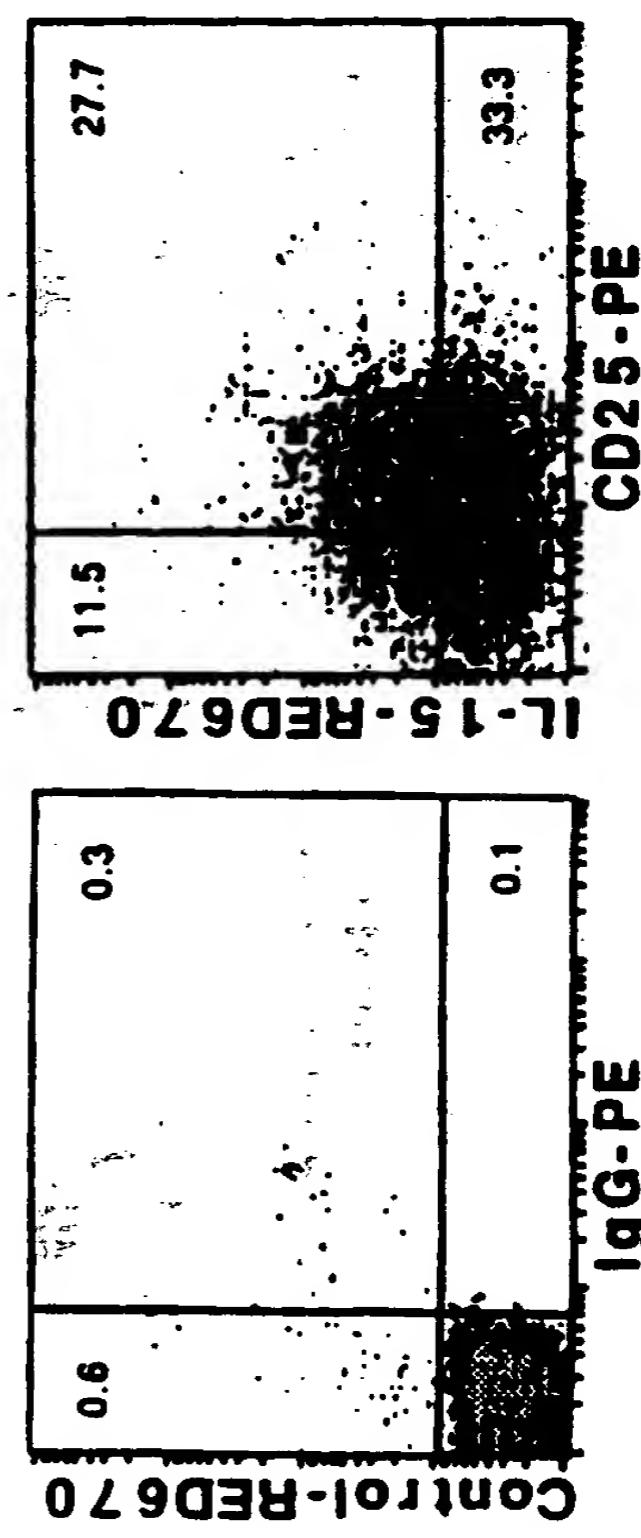
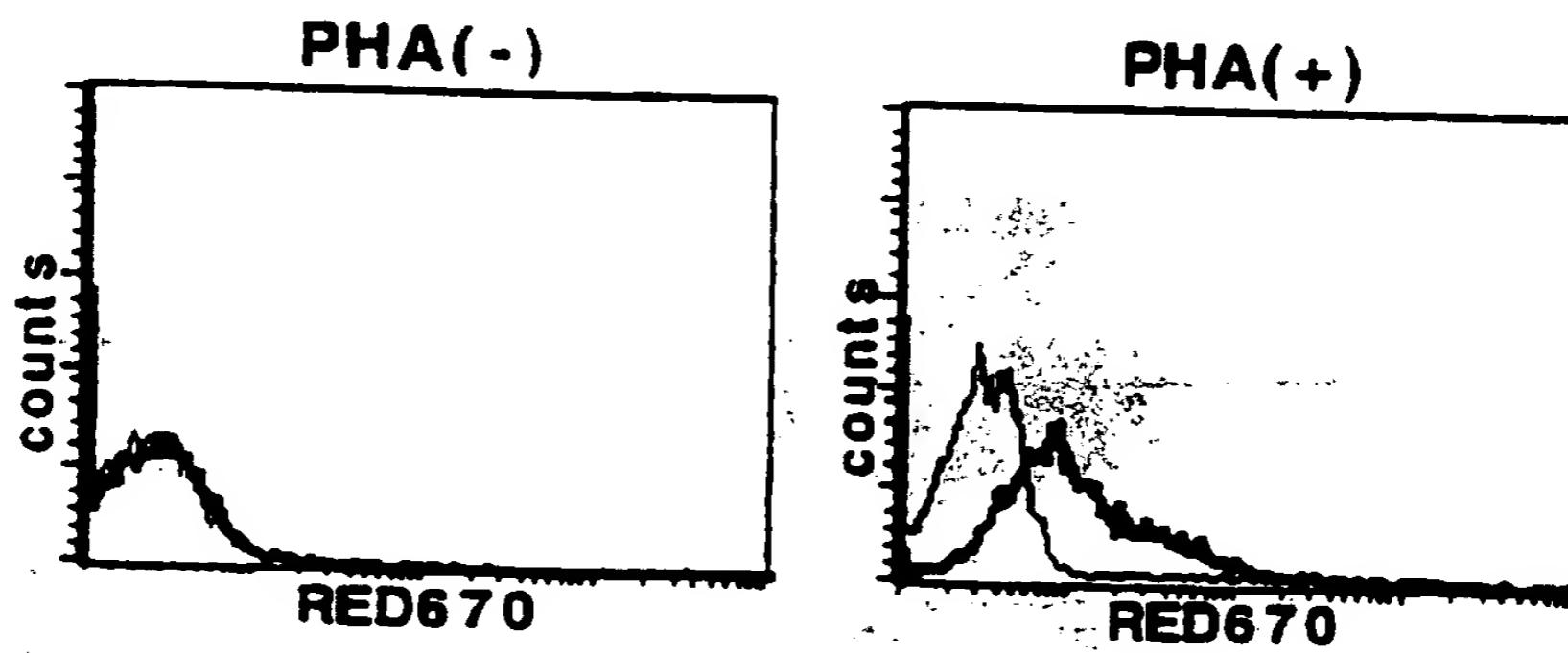
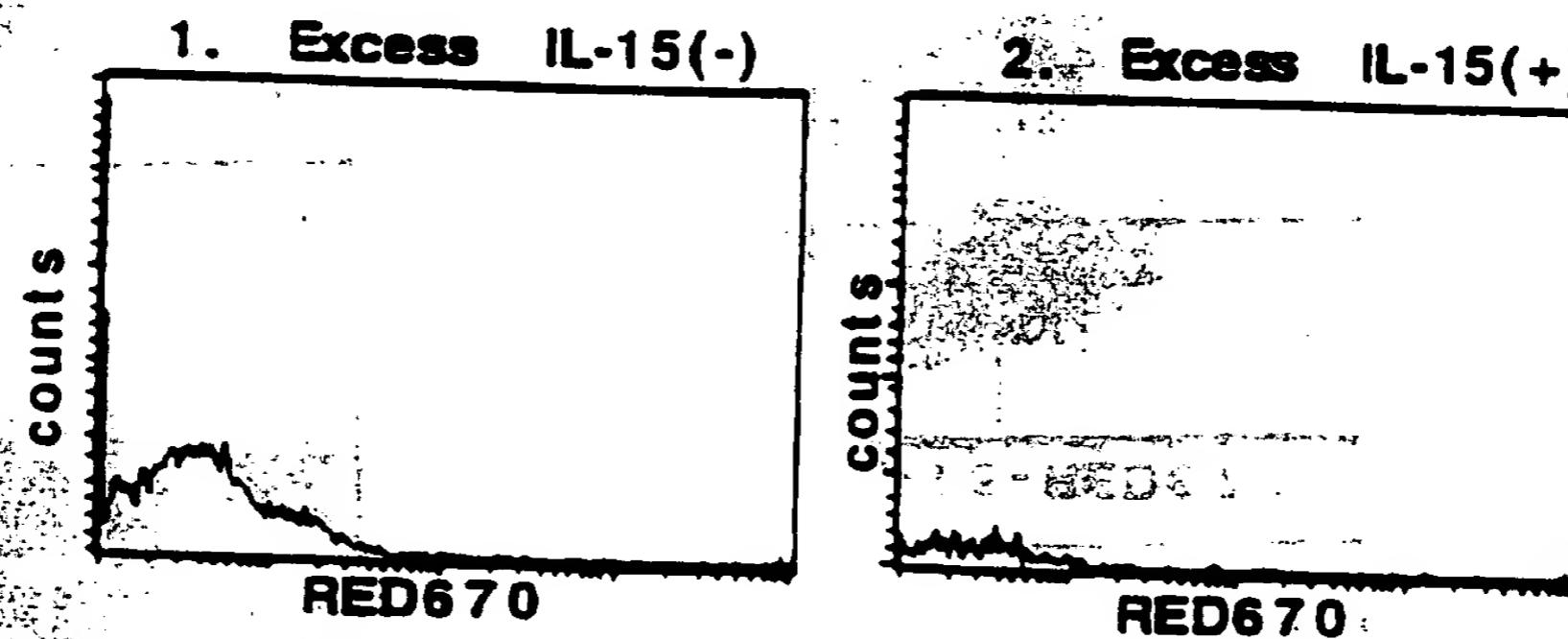


FIG. 5A

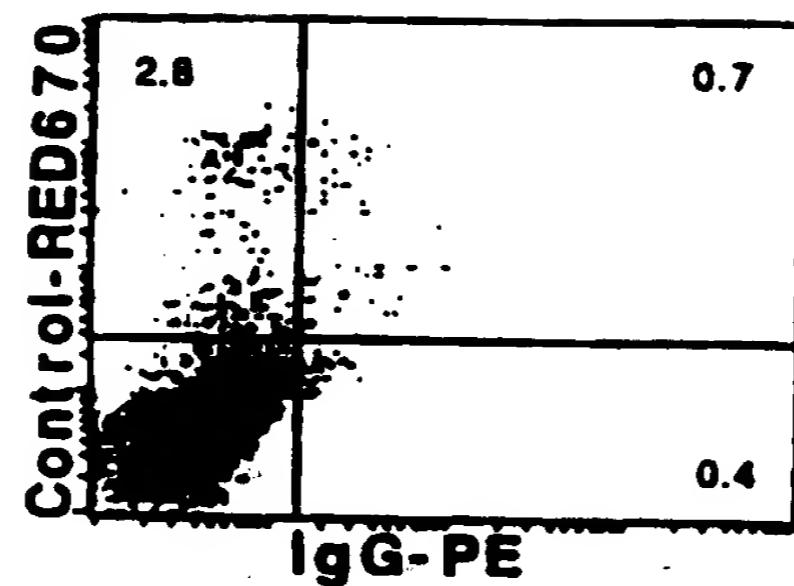


3/18

**FIG. 4A****FIG. 4B****SUBSTITUTE SHEET (RULE 26)**

6/18

PHA



CsA/ PHA

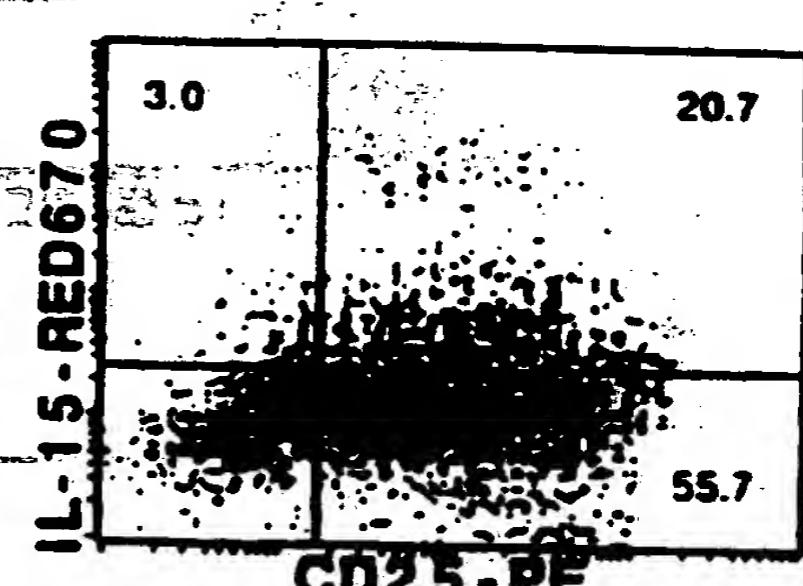
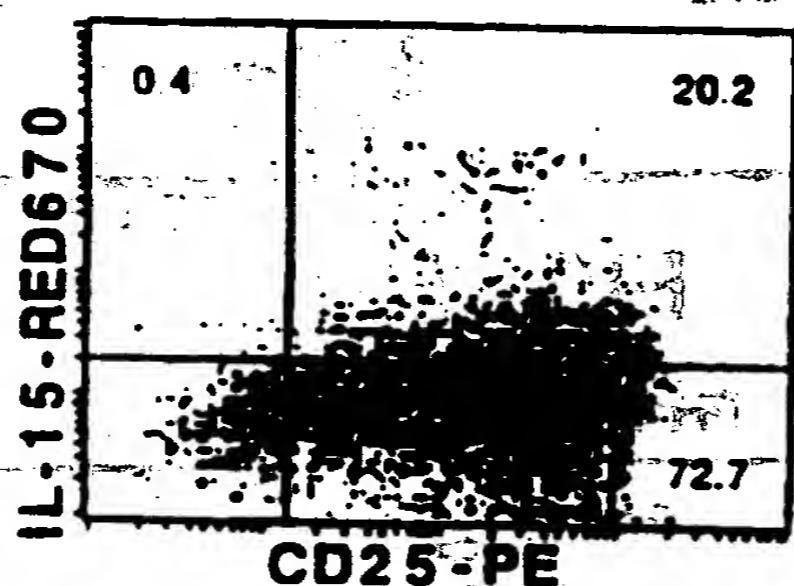
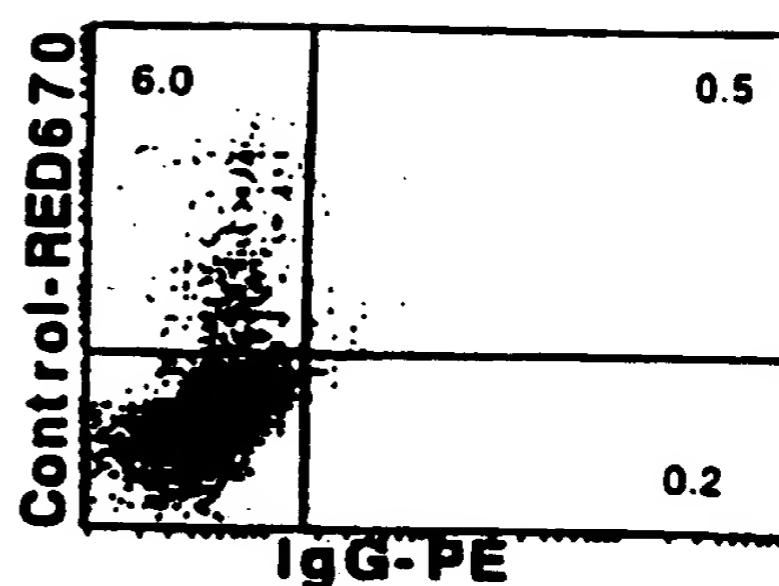


FIG. 6A

FIG. 6B

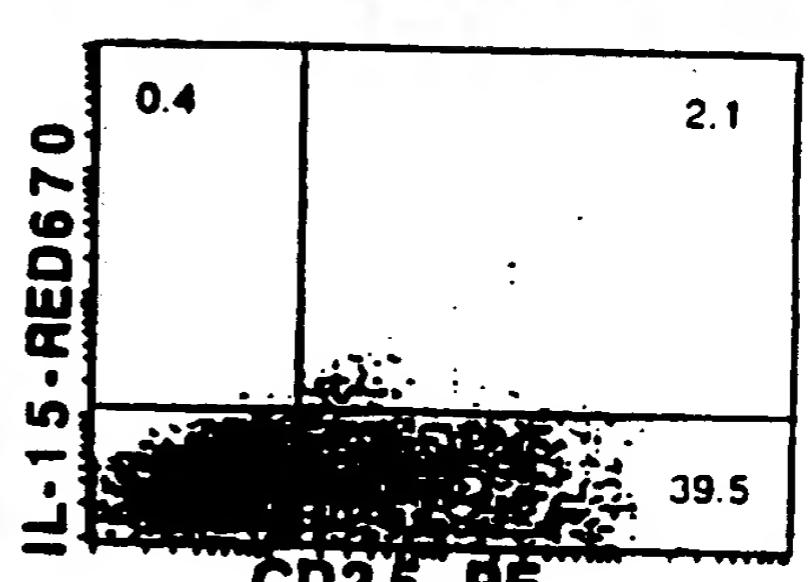
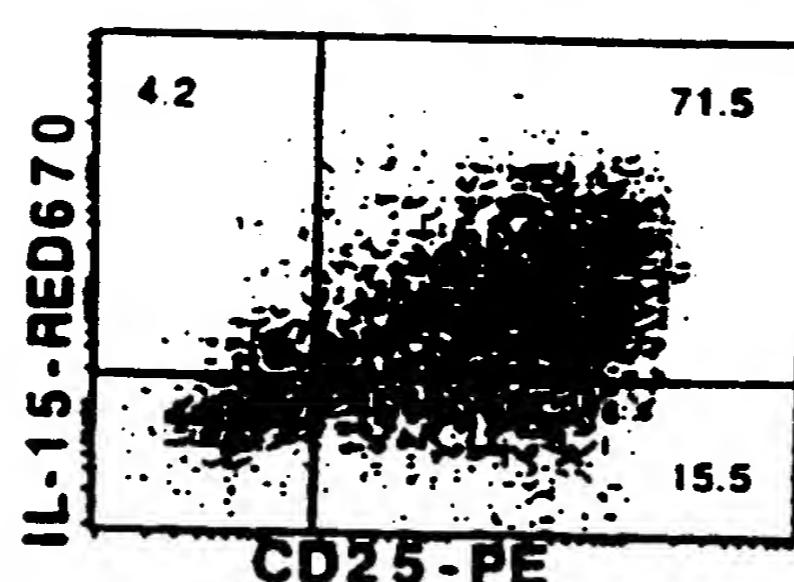
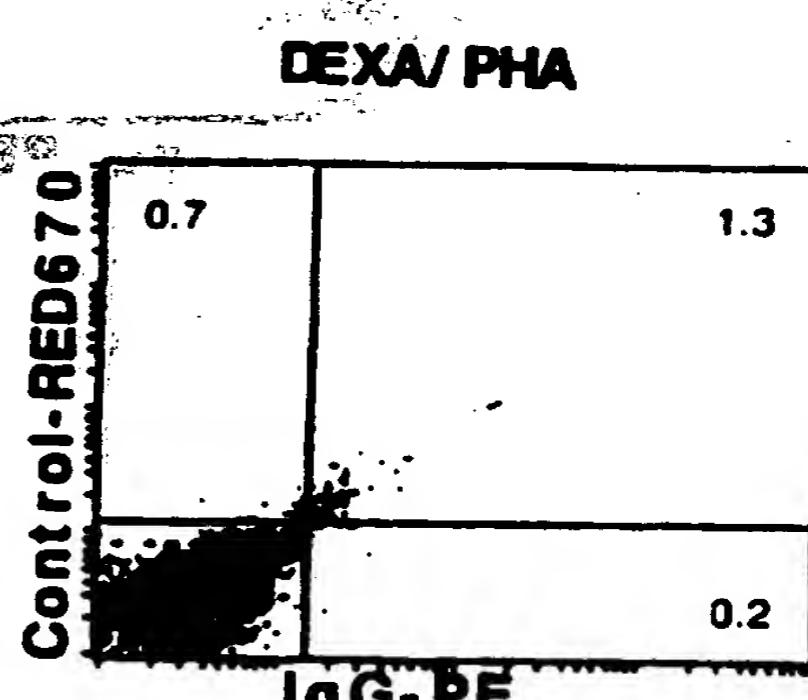
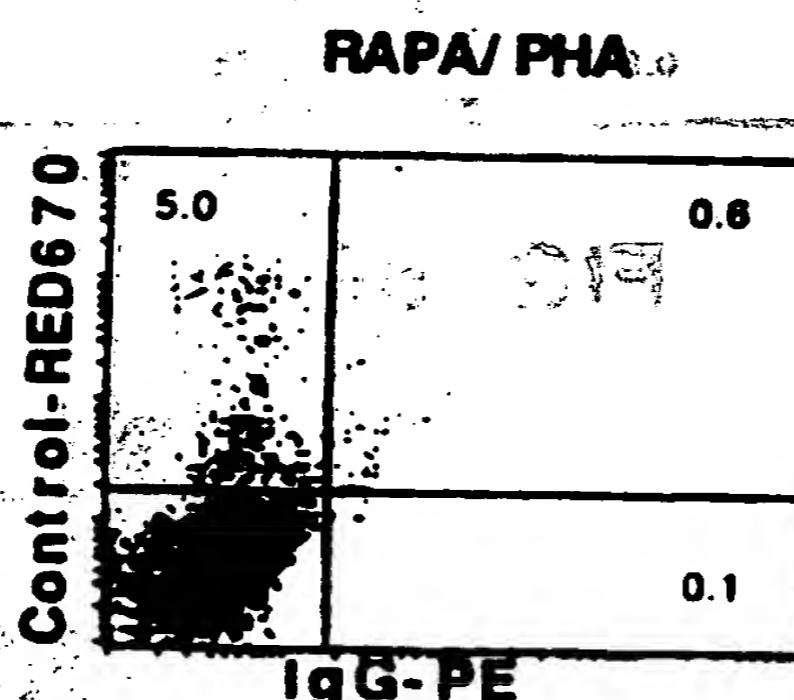


FIG. 6C

FIG. 6D

SUBSTITUTE SHEET (RULE 26)

5/18

PHA(+) for 1 Day

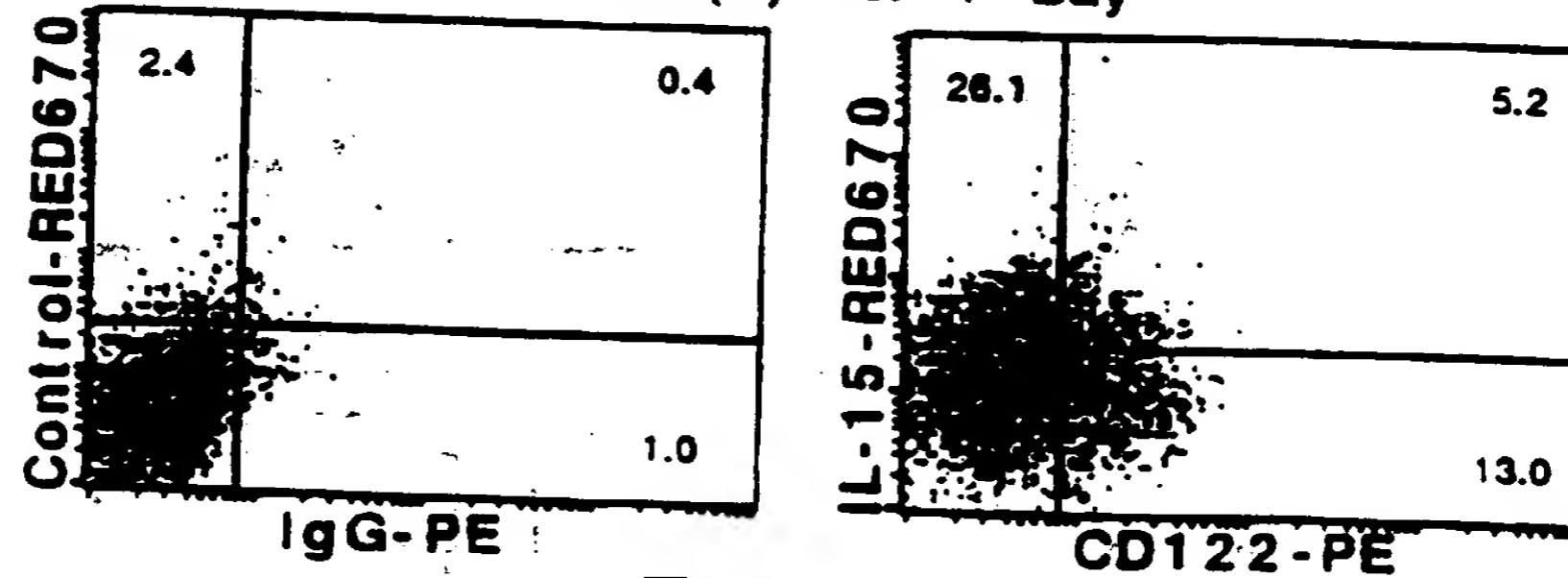


FIG. 5B

PHA(+) for 3 Days

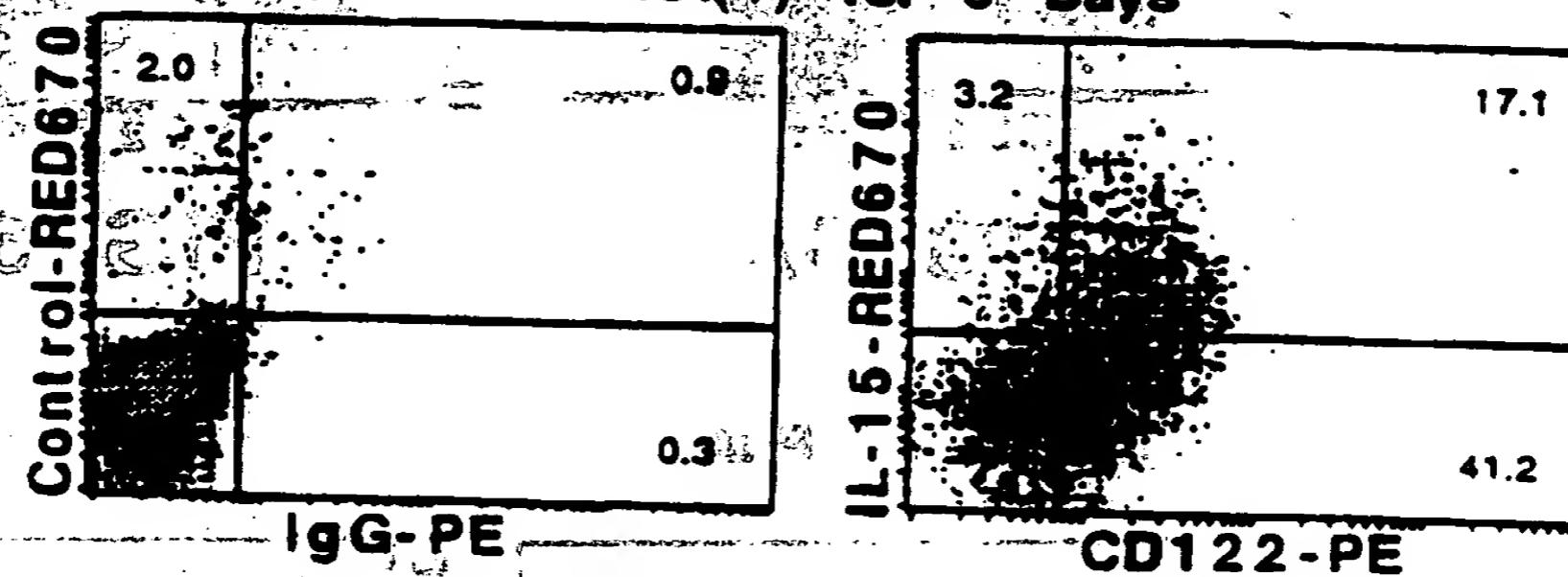


FIG. 5C

SUBSTITUTE SHEET (RULE 26)

8/18

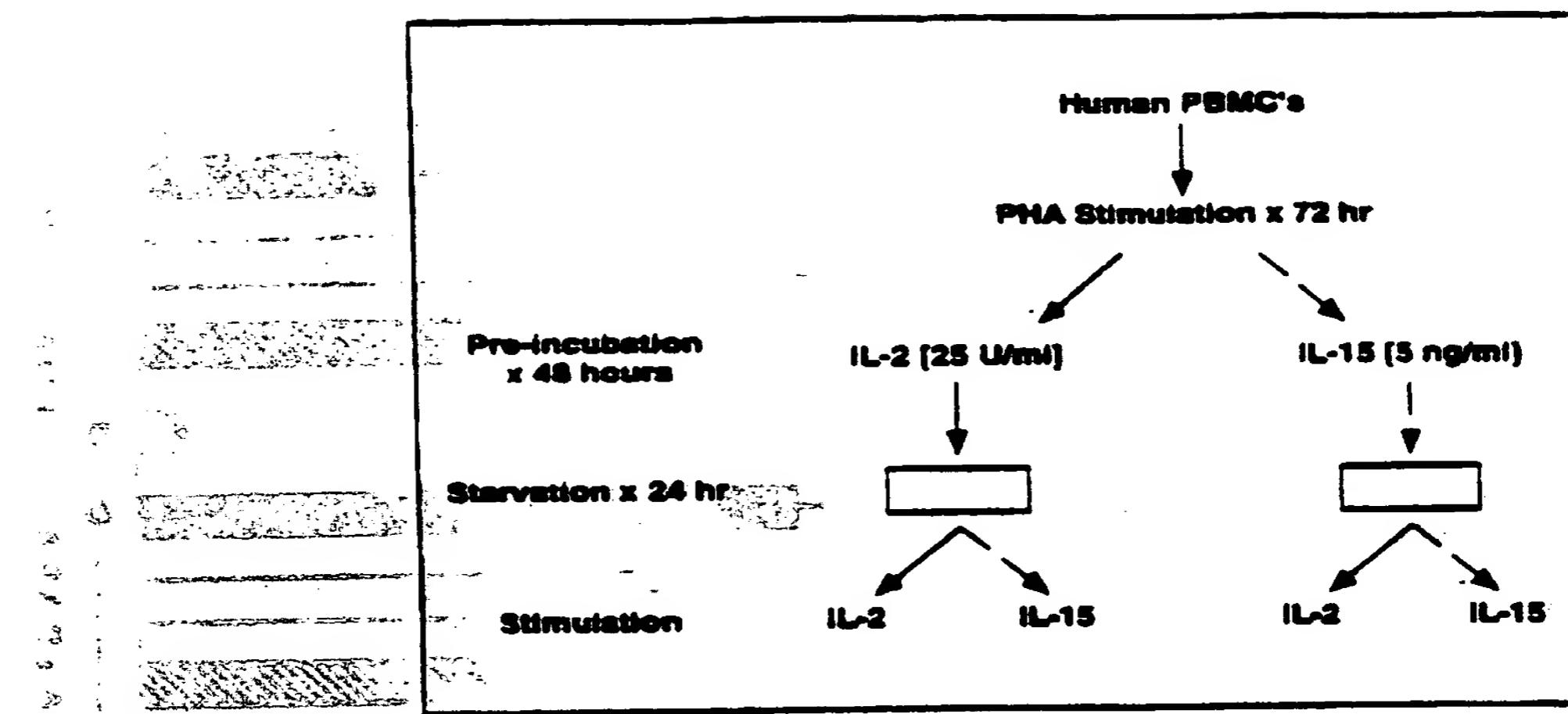


FIG. 8A

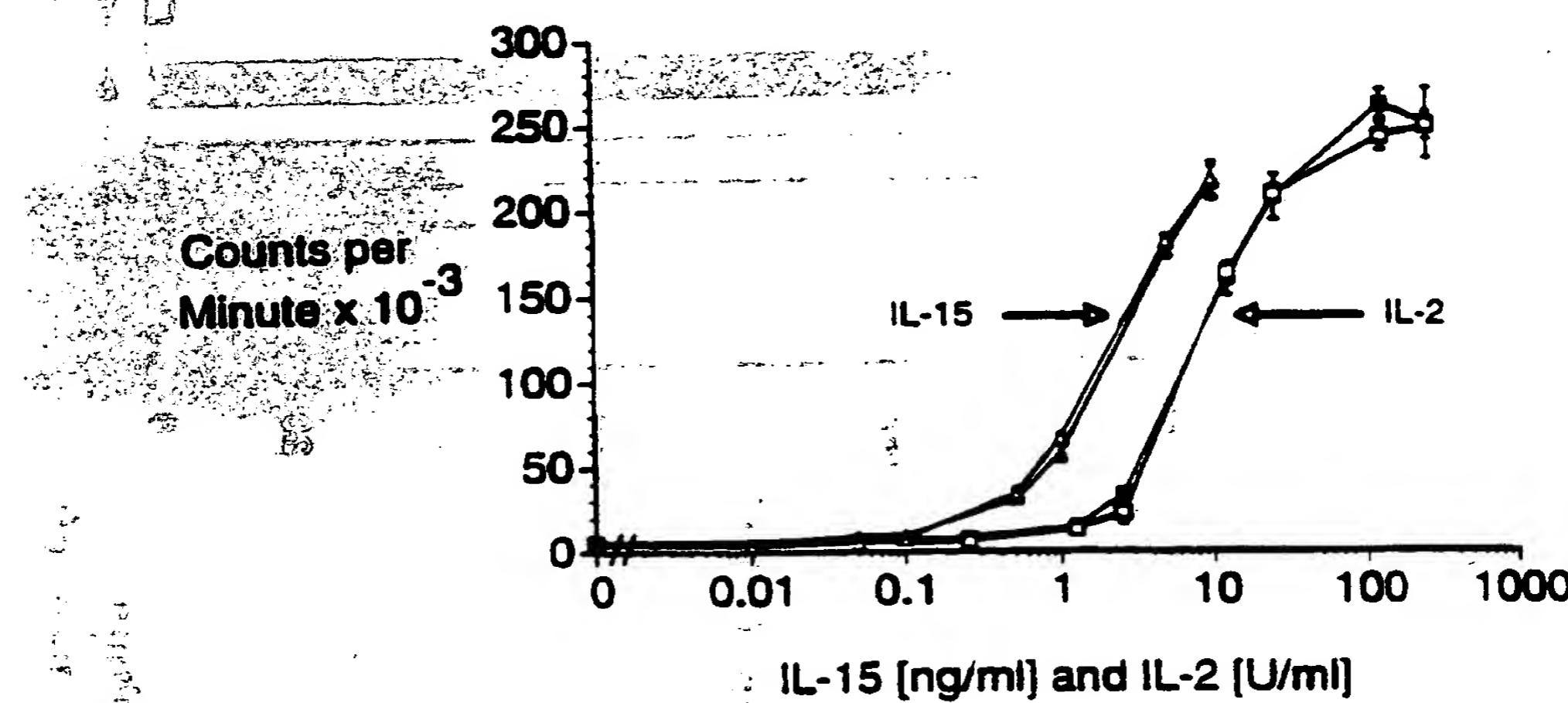


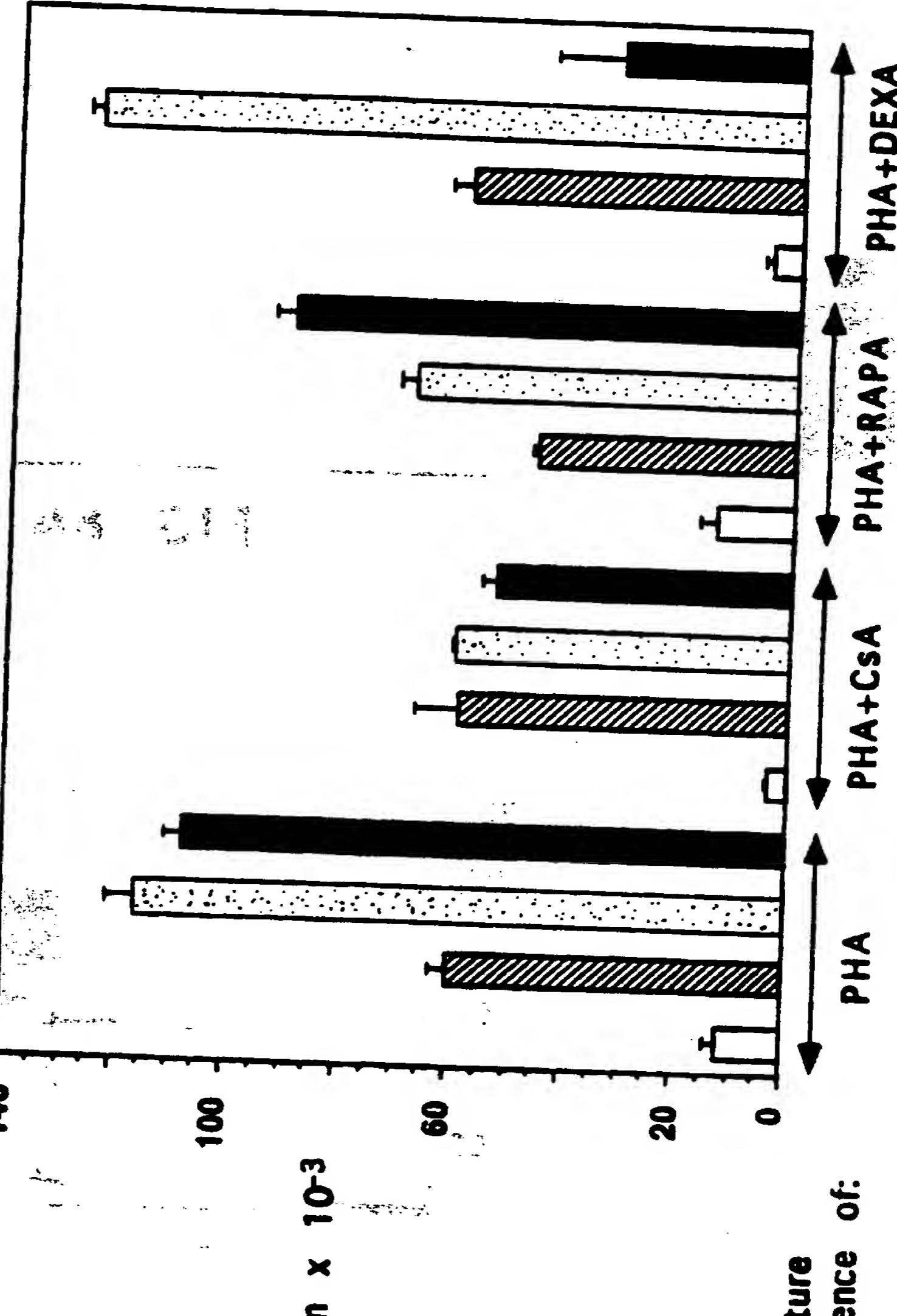
FIG. 8B

SUBSTITUTE SHEET (RULE 26)

7/18

Secondary culture
in the presence of:

none
PHA
IL-2
IL-15



Primary culture
in the presence of:

FIG. 7

10/18

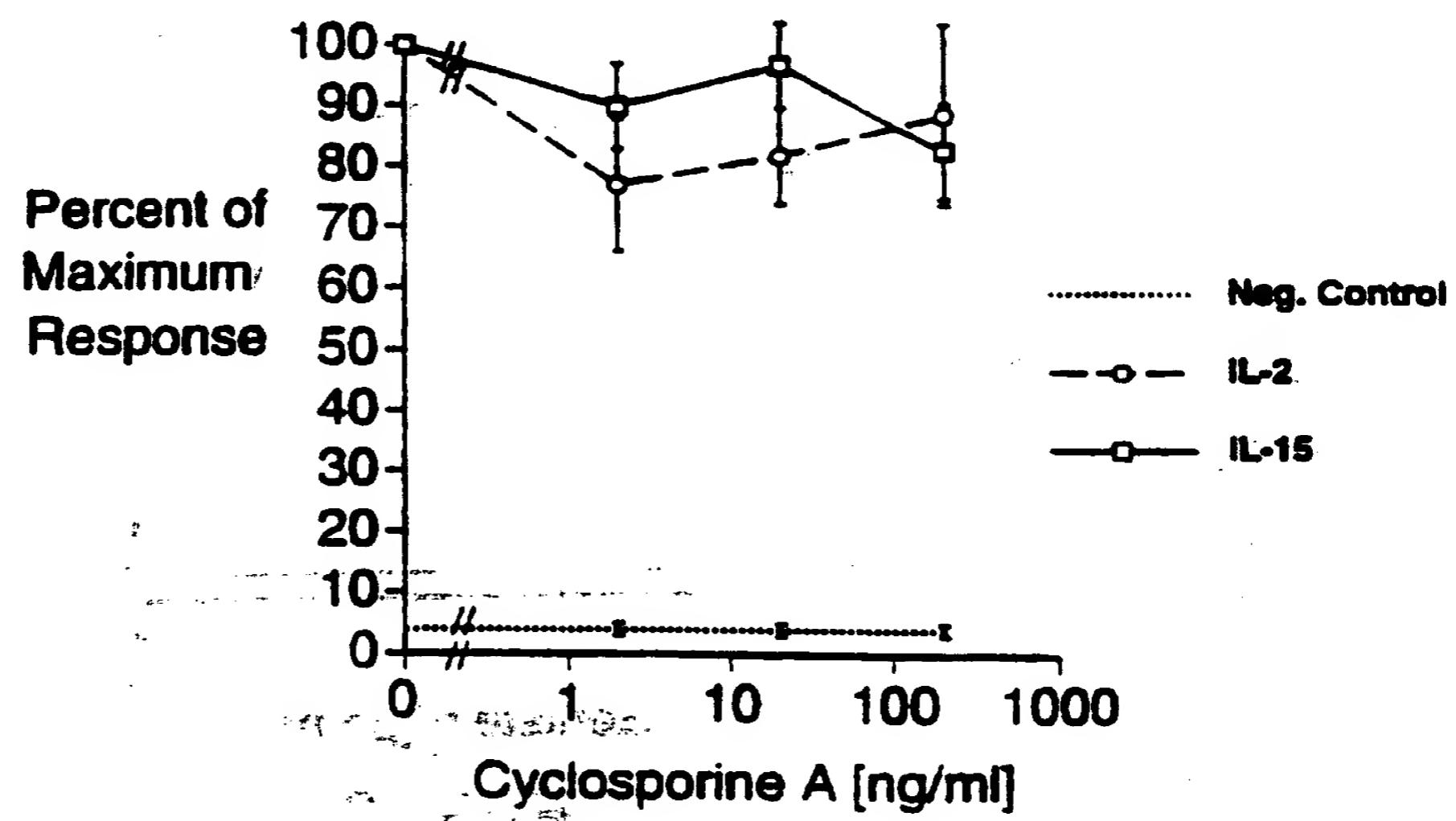


FIG. 11

SUBSTITUTE SHEET (RULE 26)

9/18

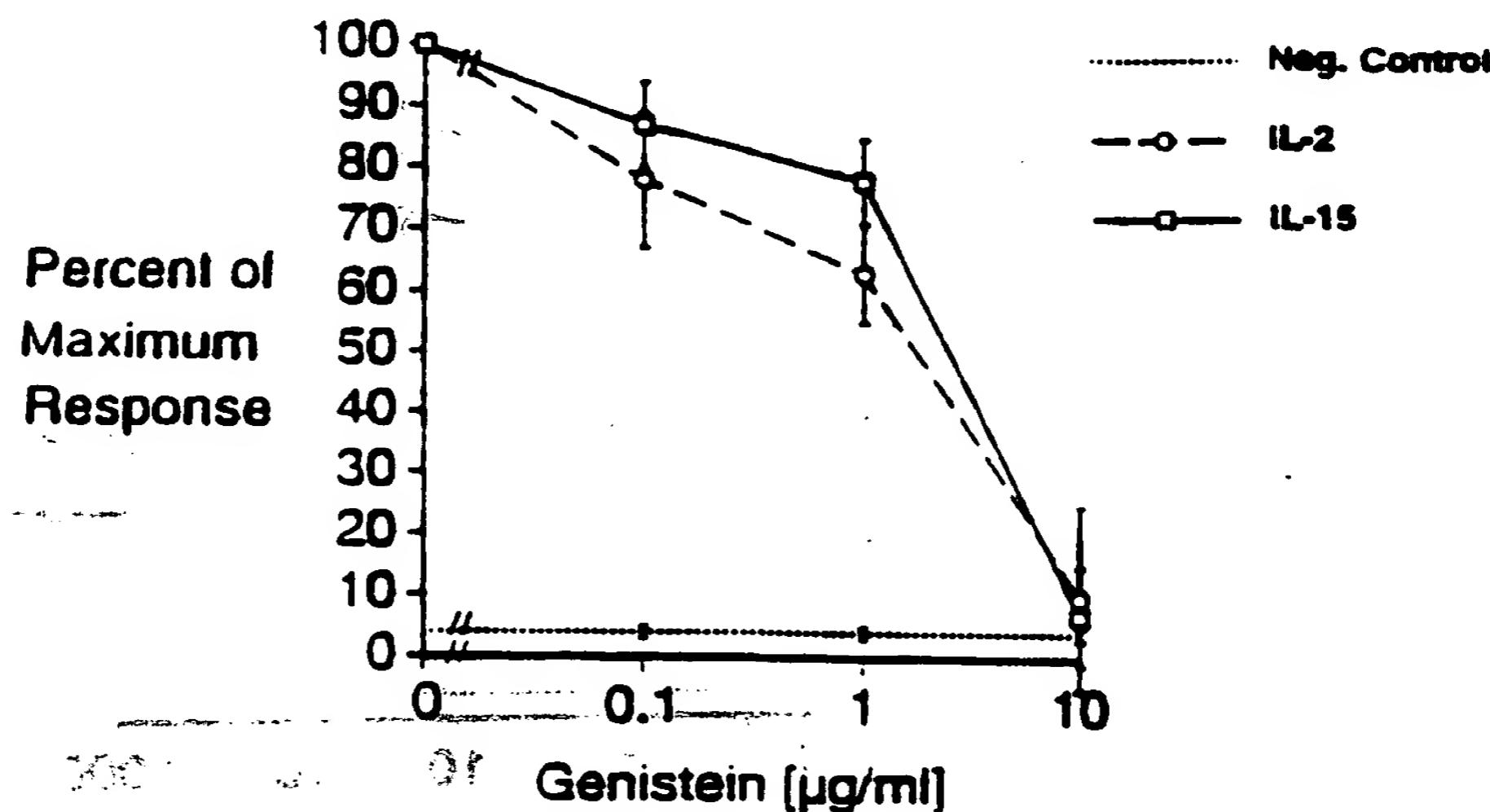


FIG. 9

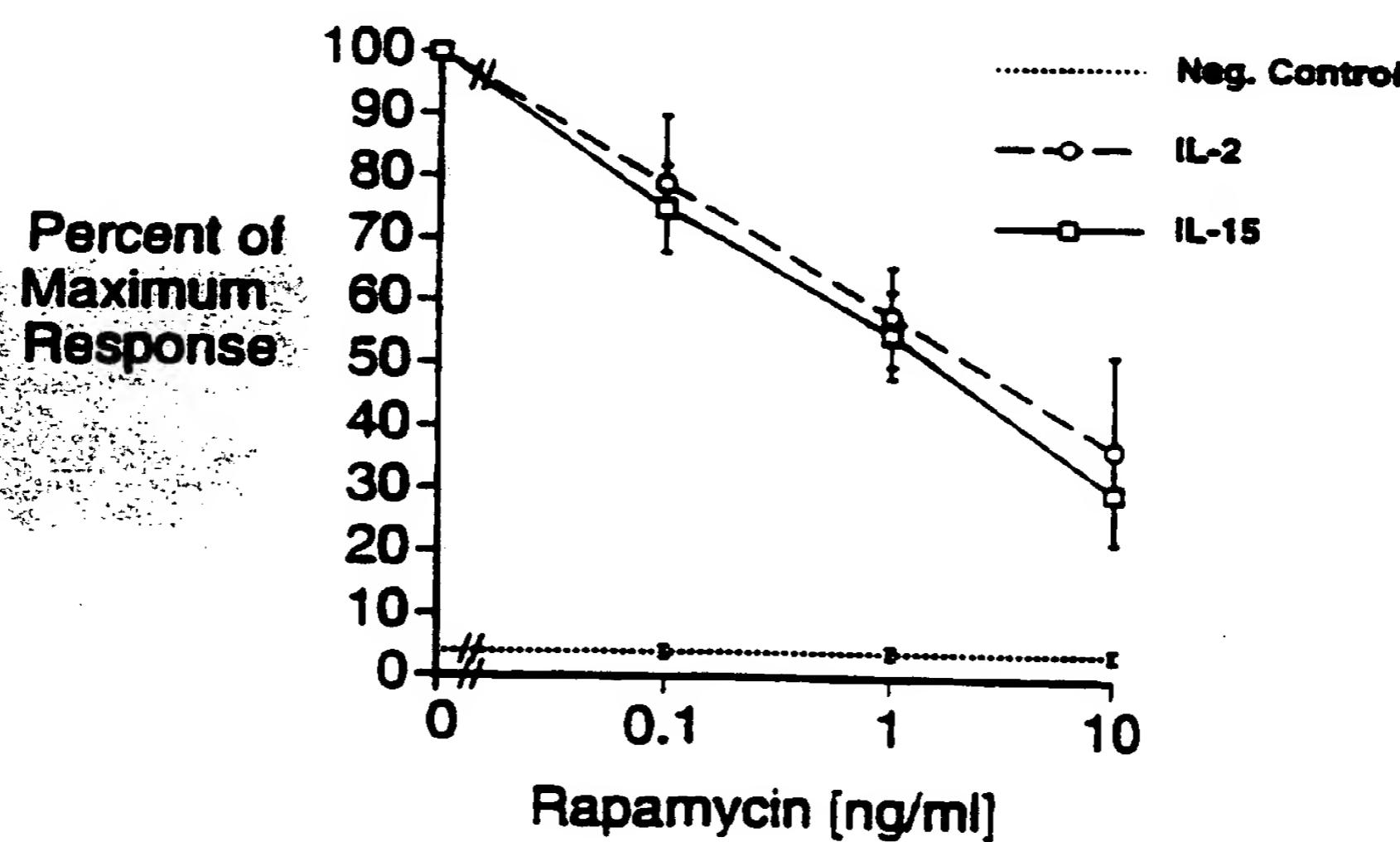


FIG. 10

SUBSTITUTE SHEET (RULE 26)

12/18

DNA Strider™ 1.1 #### Friday, February 2, 1996 9:16:01

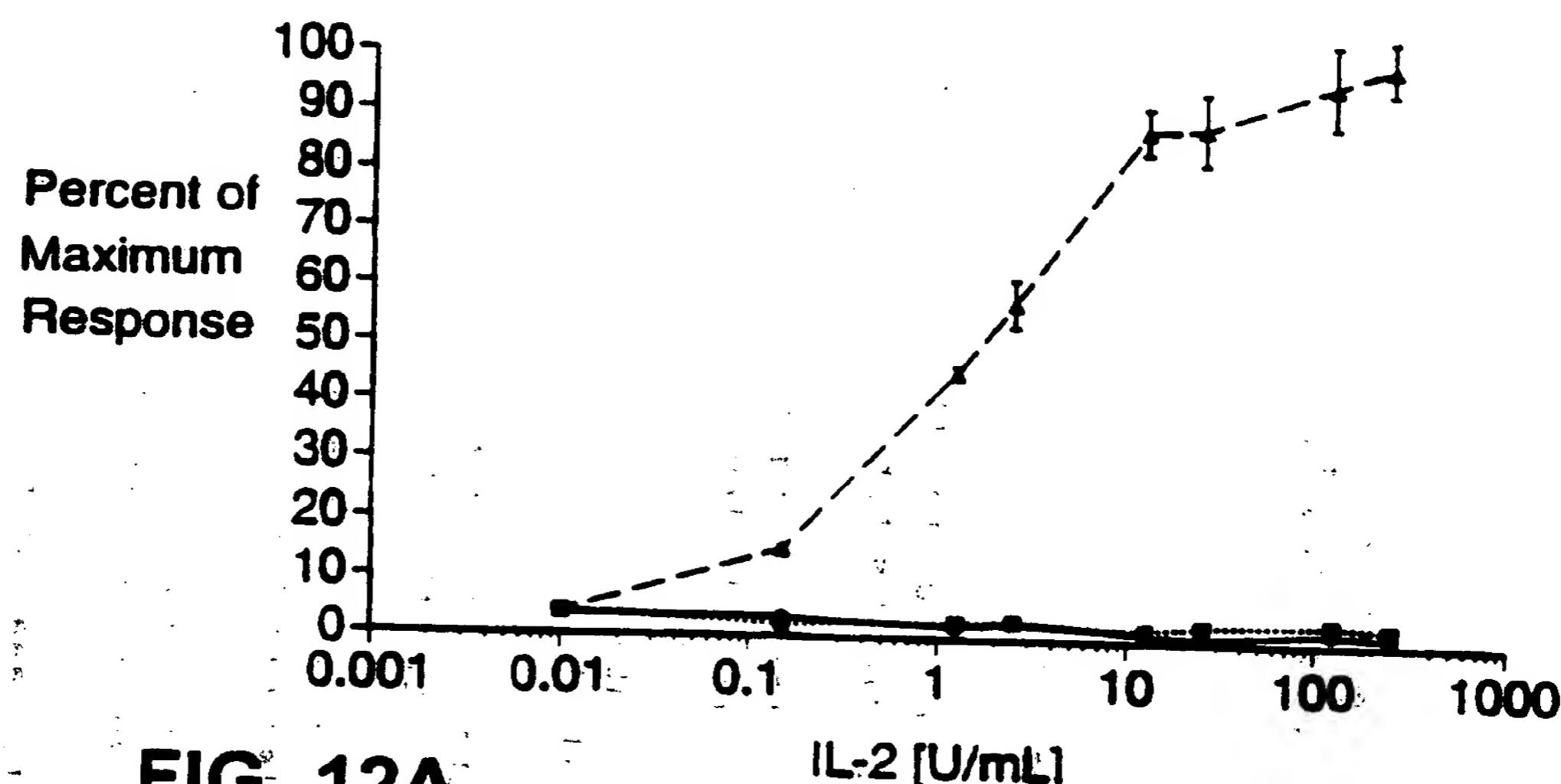
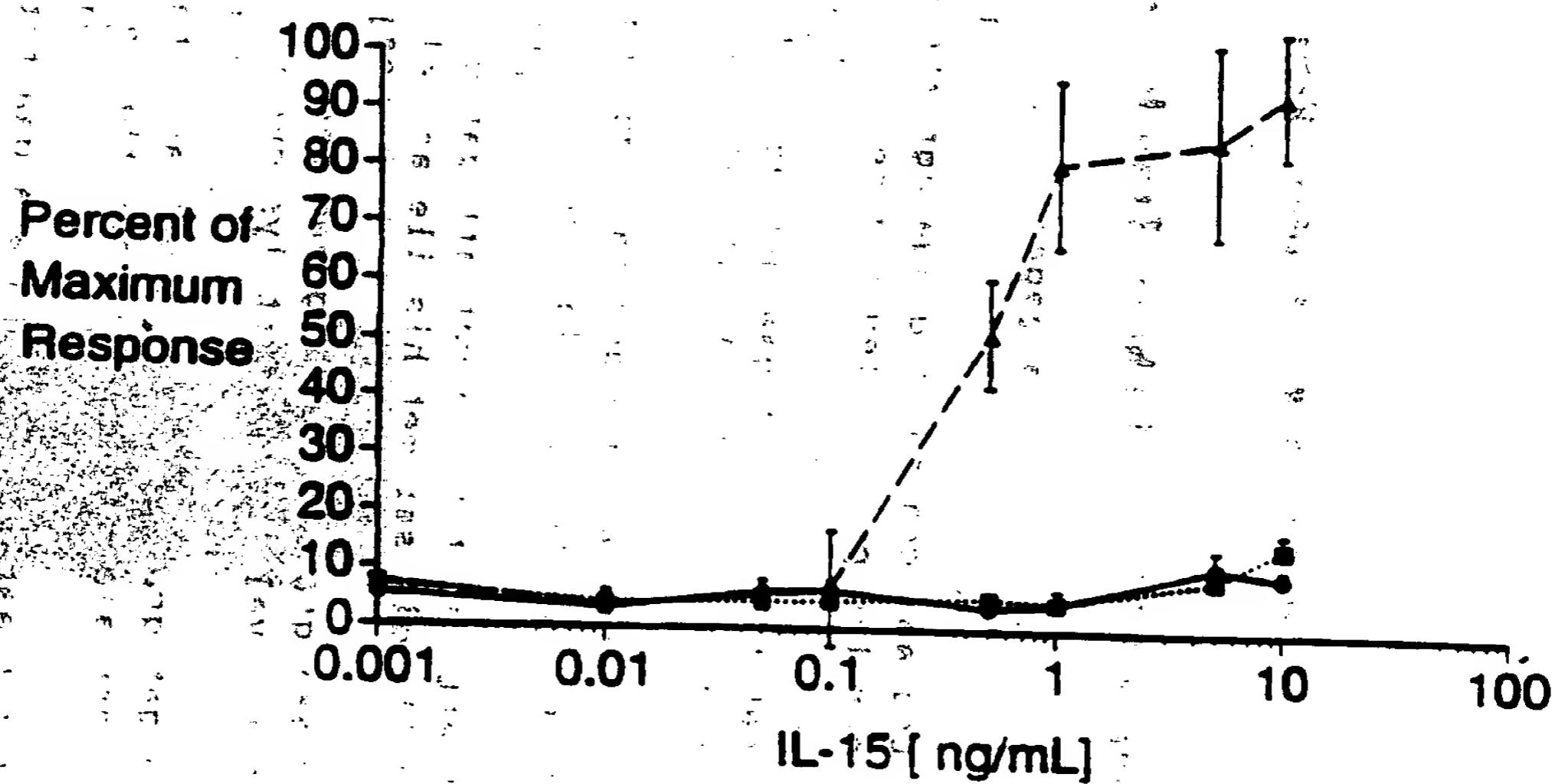
hIL-15/Immunex/ORE → 1-phase Translation

DNA sequence	b.p.	atgagaatttcg . . . aacactttcttga	linear
1/1	489	atgagaatttcg . . . aacactttcttga	linear
atg aga acc tgg aaa cca cat ttg aga agt att tcc atc cag tgc tac ttg tgt ttg tta ctt Met arg ile ser lys pro his leu arg ser ile ser ile gln cys tyr leu cys leu leu	31/11		
61/21			
ctt aac agt cat ttg act gaa gct ggc att cat gtc ttg atc ttg ggc ttg tgt ttg ttc agt leu asn ser his phe leu thr glu ala gly ile his val phe ile leu gly cys phe ser	91/31		
121/41			
gca ggg ctt cct aaa aca gaa gcc aac tgg ttg aat gta atc agt gat ttg aaa aaa att ala gly leu pro lys thr glu ala asn trp val asn val ile ser asp leu lys ile	181/61		
gaa gat ttg att caa tct atg cat att gat gct act ttg ttt ttt acg gaa agt gat gtt cac glu asp ile leu ser met his ile asp ala thr ile tyr thr glu ser asp val his	211/71		
241/81			
ccc agt tgc aaa gta aca gca atg aag ttt ttg gag ttg ctt ctc ttg gag ttg ctt att tca ctt pro ser cys lys val thr ala met lys cys phe leu glu leu gln val ile ser leu	271/91		
301/101			
gag tcc gga gat gca agt att cat gat aca gaa aat ttg atc atc cta gca aac aac glu ser gly asp ala ser ile his asp thr val glu asn ile leu ala asn asn	331/111		
361/121			
agt ttg tct tct aat ggg aat gta aca gaa tct gga tgc aaa gaa ttg gag gaa ttg gag ser leu ser ser asn gly asn val thr glu ser gly cys lys glu glu leu glu	391/131		
421/141			
gaa aaa aat att aaa gaa ttt ttg cag agt ttg gta cat att gtc caa atg ttg atc aac glu lys asn ile lys glu phe leu gln ser phe val his ile val gln met phe ile asn	451/151		
481/161			
act tct tga thr ser RPA			

SUBSTITUTE SHEET (RULE 26)

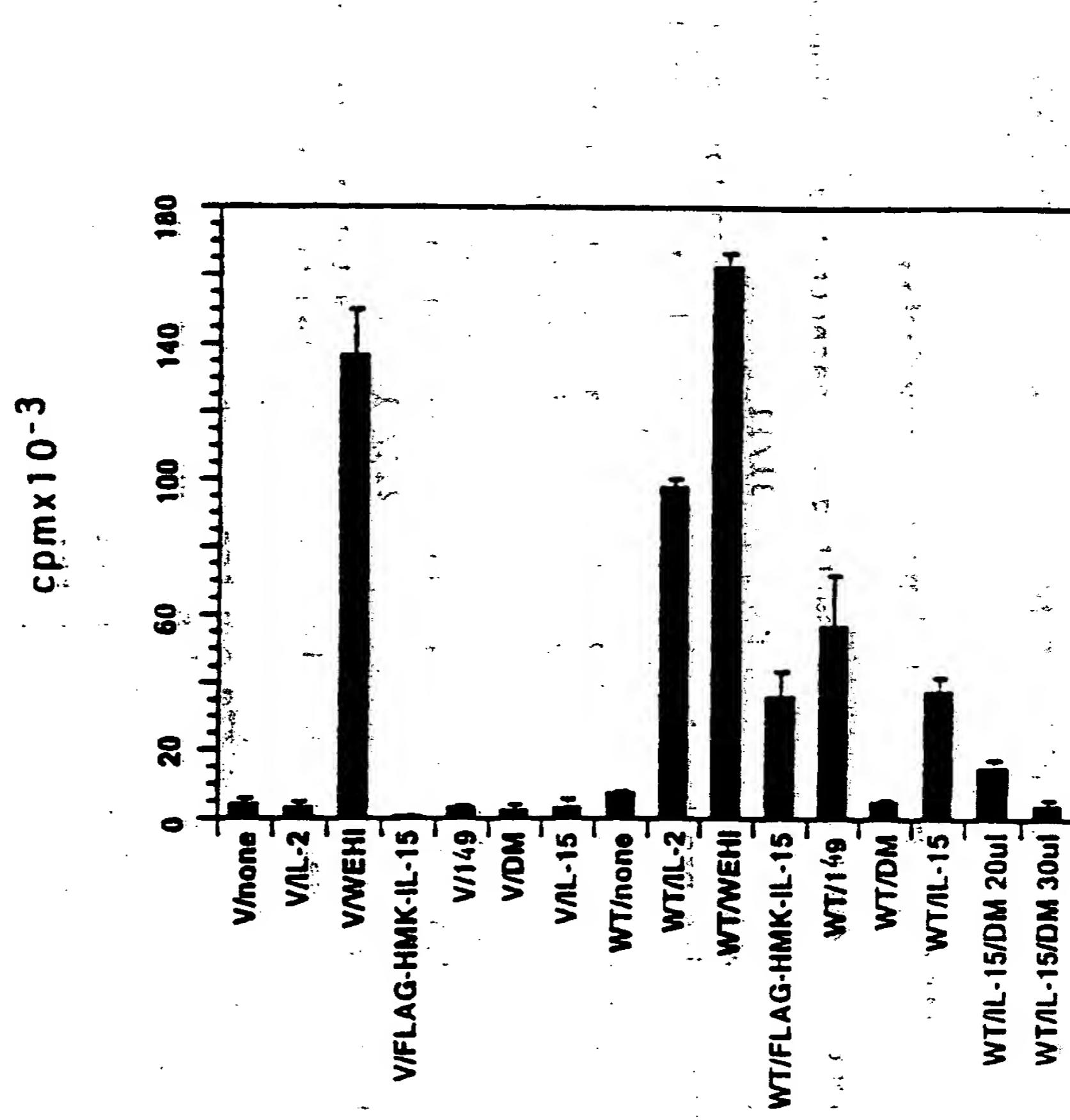
FIG. 13

11/18

**FIG. 12A****FIG. 12B****SUBSTITUTE SHEET (RULE 26)**

14/18

FIG. 15



13/18

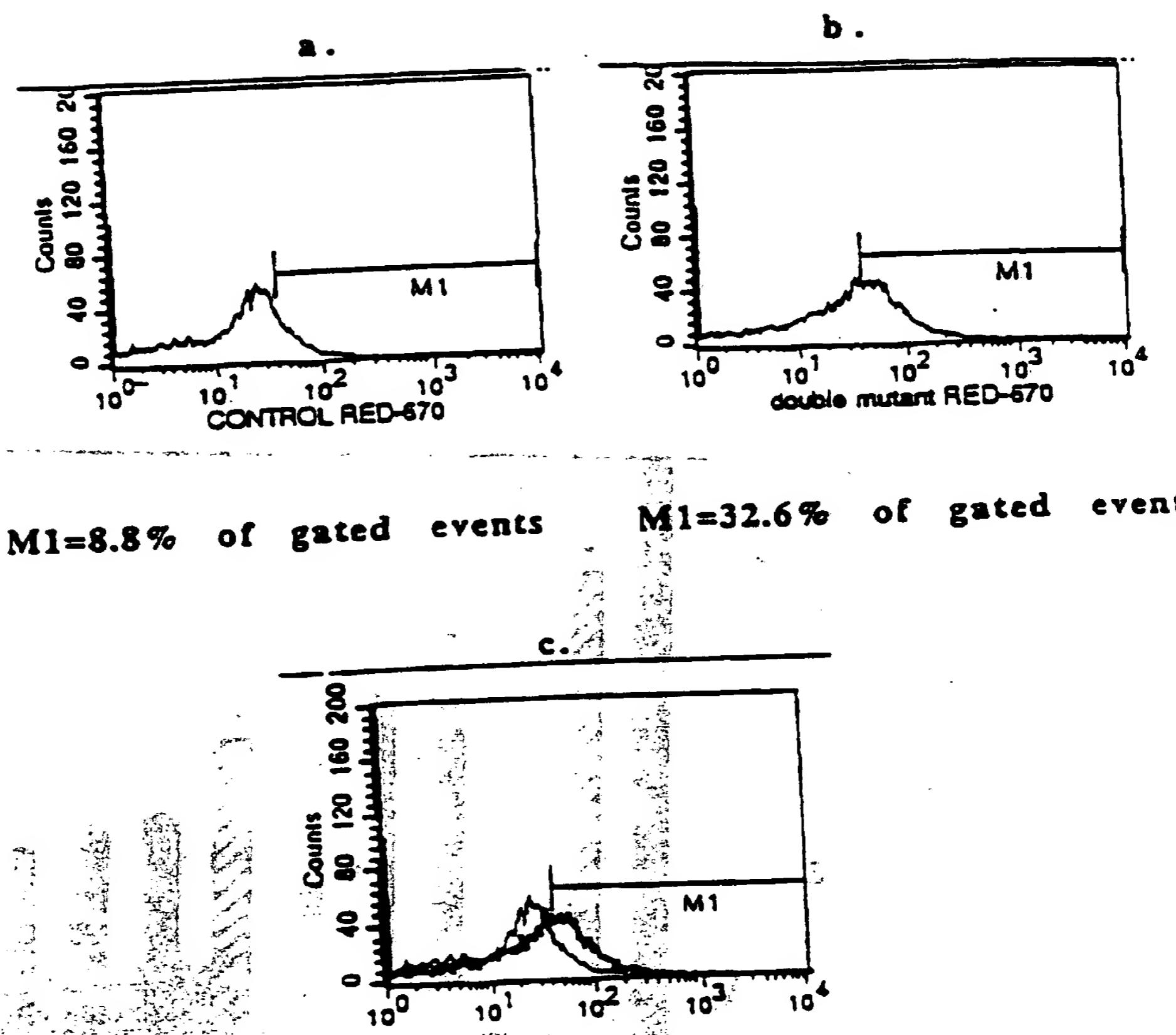
DNA Strider™ 1.1 ## Friday, February 2, 1996 9:24:40

human IL-15/169/176 mutant -> 1-phase Translation

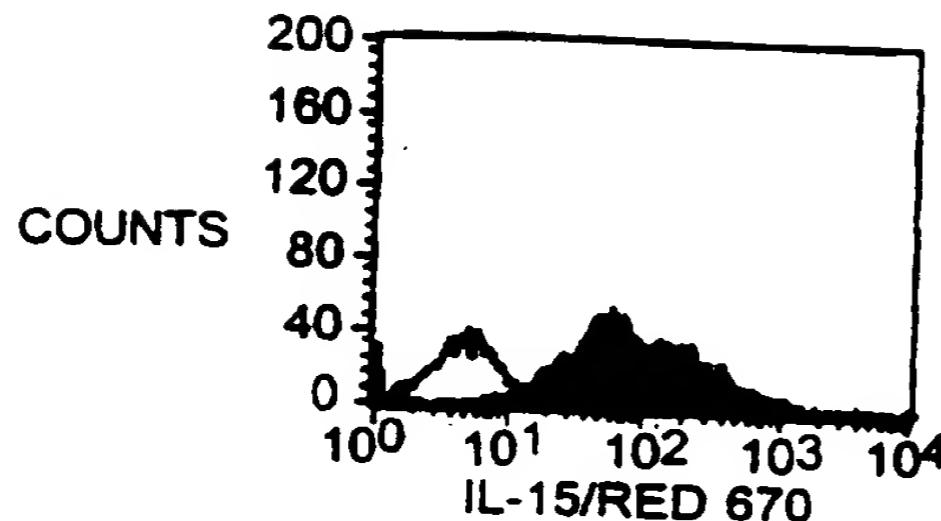
DNA sequence	489 b.P.	atgagaatttcg . . . aacacttcttga	linear
1/1			
atg aga att tcg aaaa cccca ccc ttg aga aat ccc atc ccc	31/11	tgc tac ttg tgt tta ctt	
Met arg ile ser lys pro his leu arg ser ile ser ile gln	91/31	cys tyr leu cys leu leu	
61/21			
ctt aac agt cat ttt cta act gaa gct ggc att cat gtc ttc	121/41	att ttg ggc tgt ttc agt	
leu asn ser his phe leu thr glu ala gly ile his val phe	151/51	ile leu gly cys phe ser	
181/61			
gca ggg ctt cct aaa aca gaa gcc aac tgg ttg aat gta ata	211/71	agt gat ttg aaa aaa att	
ala gly leu pro lys thr glu ala asn trp val asn val ile	241/81	ser asp leu lys lys ile	
gaa gat ctt att cca tct atg cat att gat gct act tta	271/91	tat acg gaa agt gat gtt cac	
glu asp leu ile gln ser met his ile asp ala thr leu tyr	301/101	thr glu ser asp val his	
331/111			
ccc agt tgc aaa gta aca gca atg aag tgc ttg etc ttg gag	361/121	tta caa gtt att tca ctt	
pro ser cys lys val thr ala met lys cys phe leu glu leu	391/131	gln val ile ser leu	
391/131			
gag tcc gga gat gca agt att cat gac aca gaa aat ctg atc	421/141	atc cta gca aac aac	
glu ser gly asp ala ser ile his asp thr val glu asn ile	451/151	ile leu ala asn asn	
451/151			
gaa aaa aat att aaa gaa ttg ttg gac agt ttg gaa tgt gag	481/161	ctg gag	
glu lys asn ile lys glu ser gly cys lys glu glu leu glu	act tct tga	atc aac	
481/161	thr ser OPA	ser phe val his ile val asp met phe ile asn	

16/18

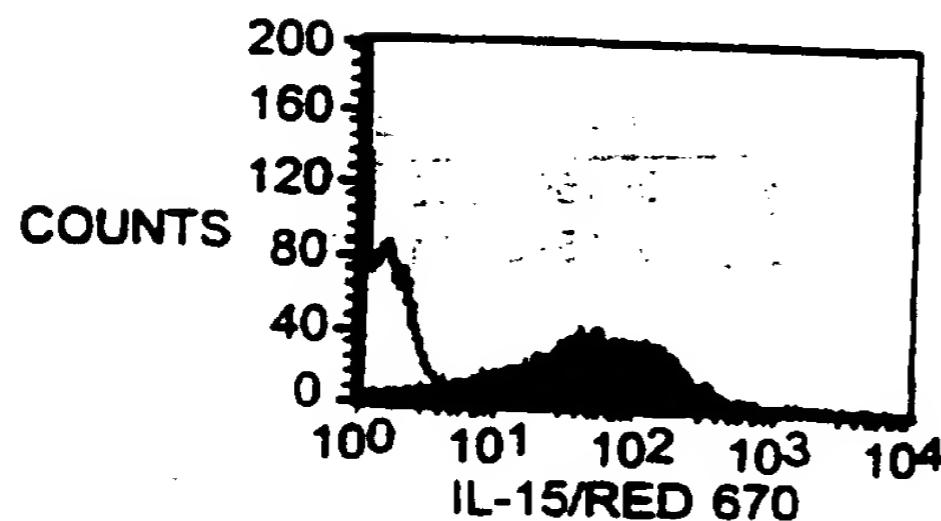
FIG. 17



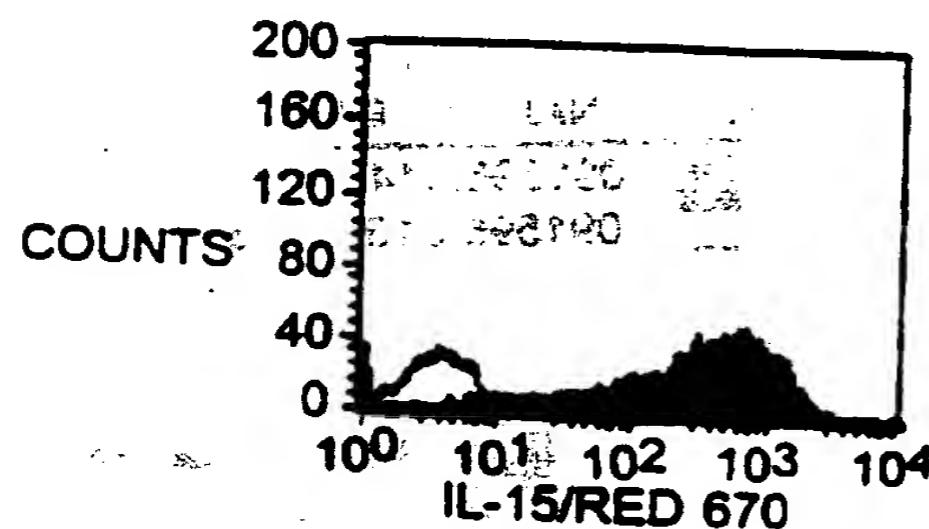
17/18



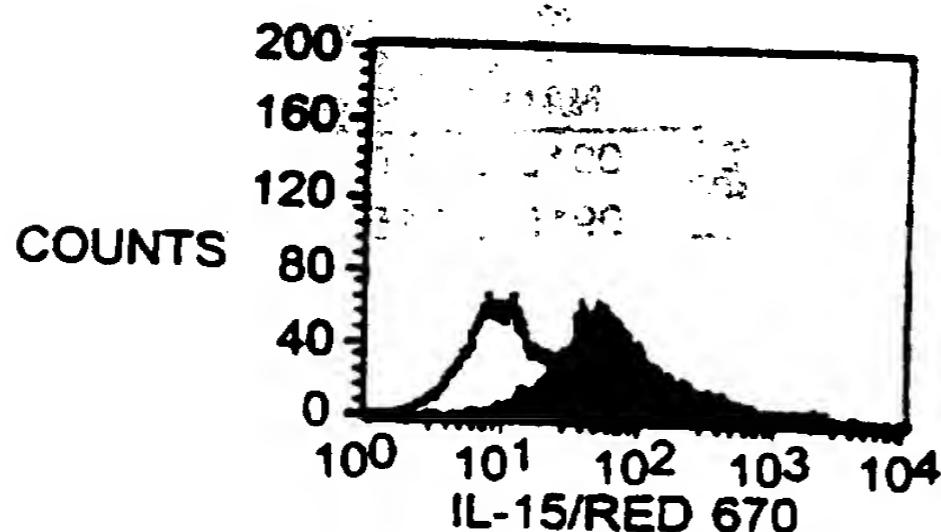
KEY	NAME	PARAMETER	GATE
■	091595.002	FL3-H	NO GATE POS
—	091595.001	FL3-H	NO GATE NEG



KEY	NAME	PARAMETER	GATE
■	091595.004	FL3-H	NO GATE POS
—	091595.003	FL3-H	NO GATE NEG



KEY	NAME	PARAMETER	GATE
■	091595.006	FL3-H	NO GATE POS
—	091595.005	FL3-H	NO GATE NEG



KEY	NAME	PARAMETER	GATE
■	091595.008	FL3-H	NO GATE POS
—	091595.007	FL3-H	NO GATE NEG

FIG. 18A

SUBSTITUTE SHEET (RULE 26)

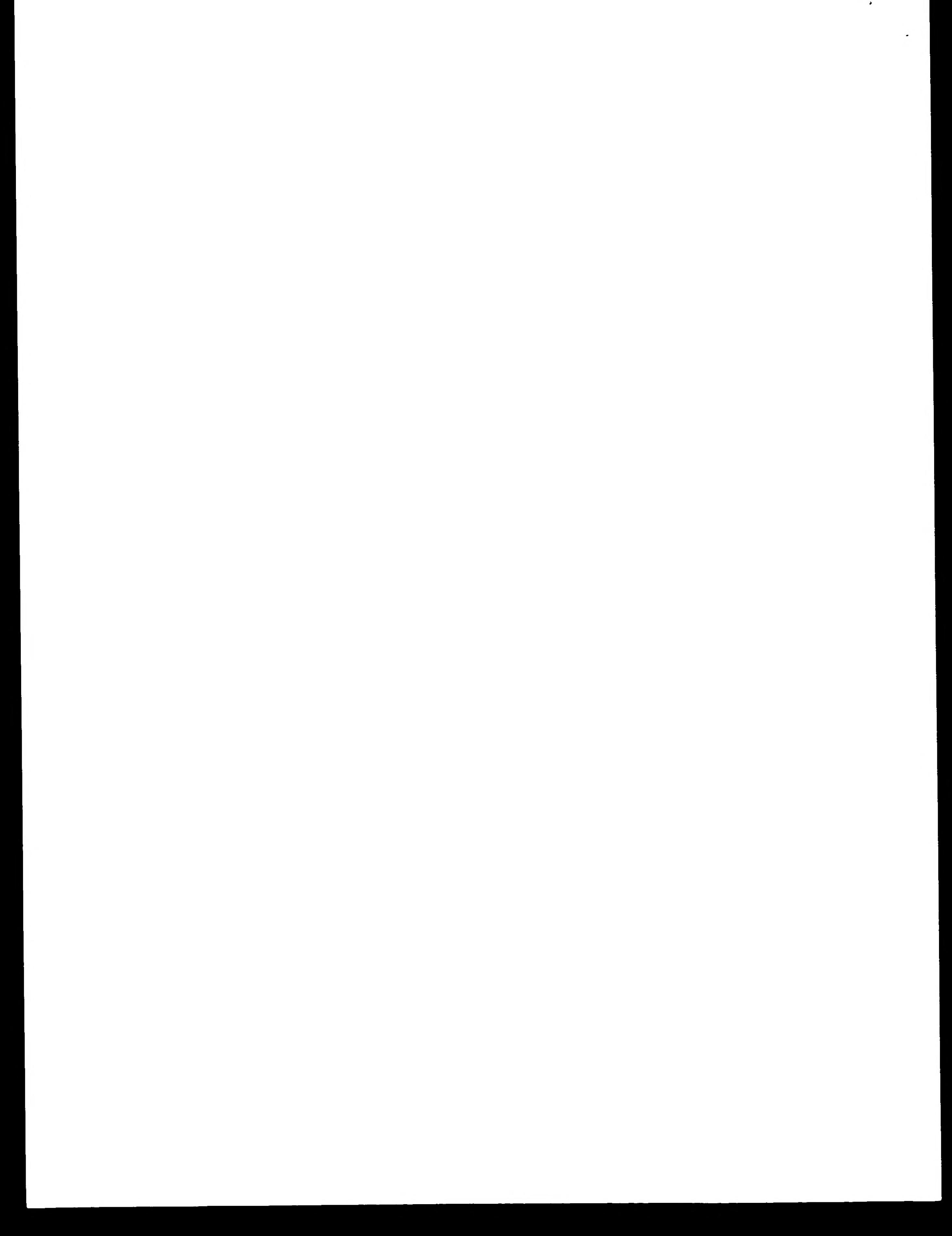
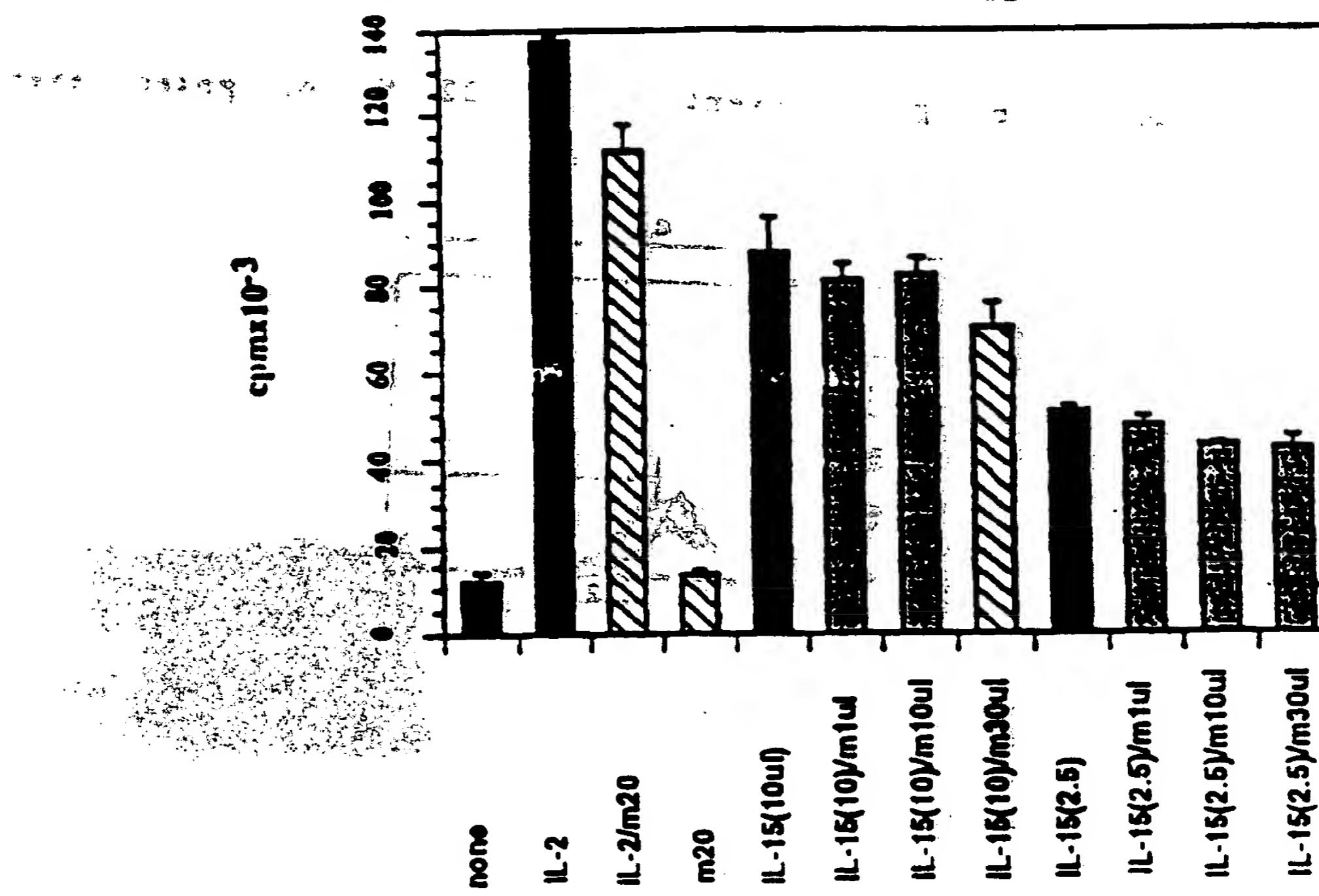
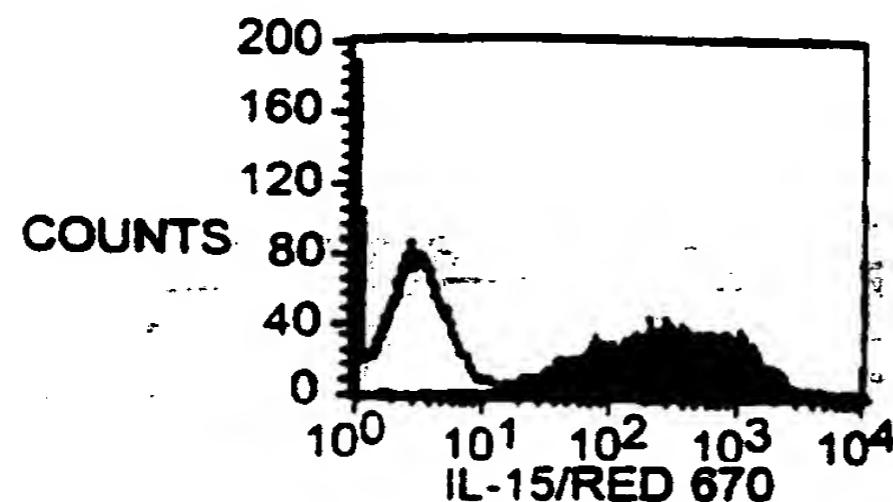


FIG. 16

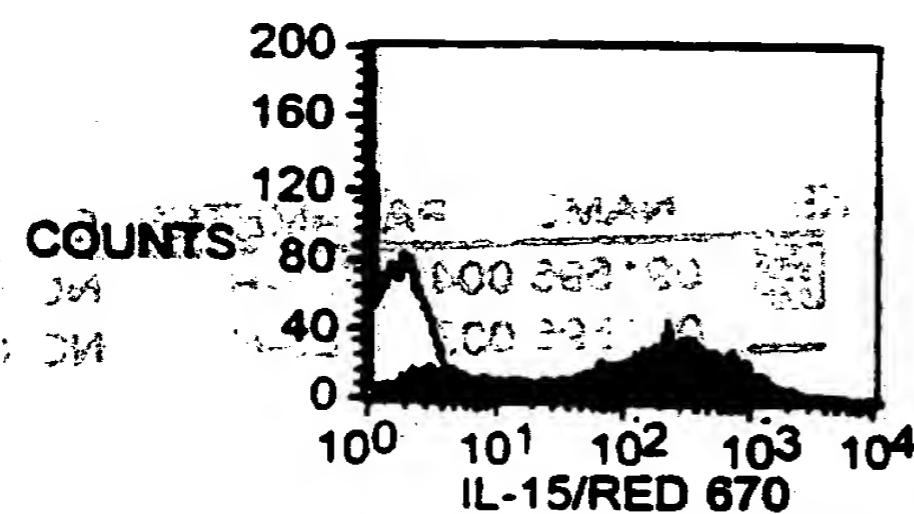
The effect of mutant IL-15 on the proliferation of PHA stimulated human PBMCS



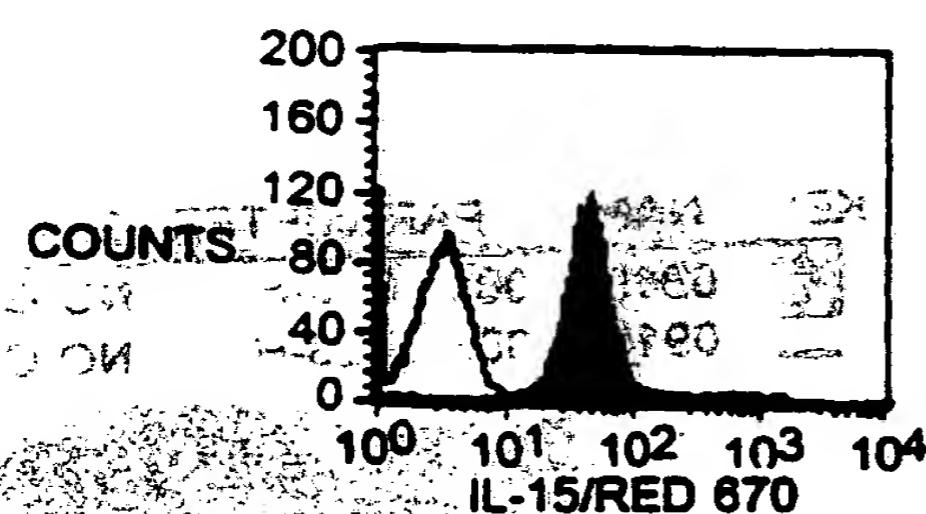
18/18



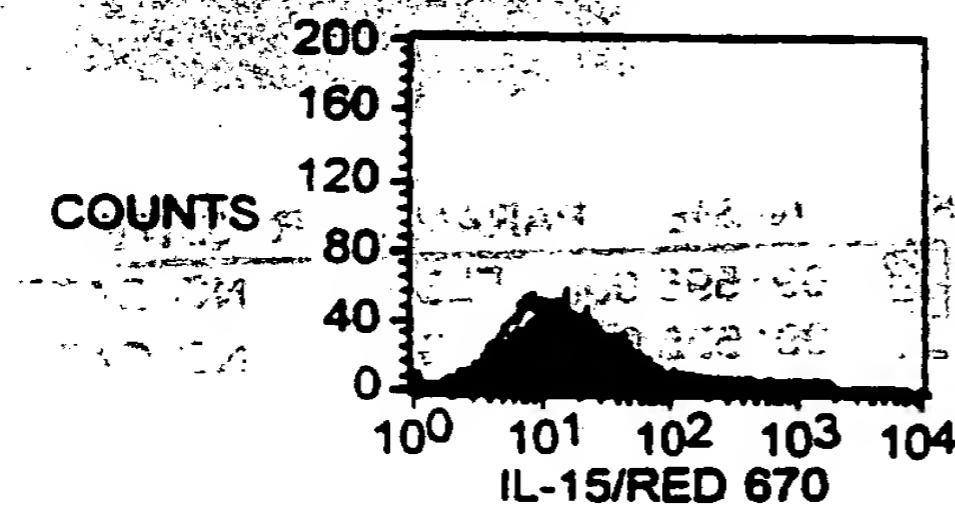
KEY	NAME	PARAMETER	GATE	
■	091595.010	FL3-H	NO GATE	POS
—	091595.009	FL3-H	NO GATE	NEG



KEY	NAME	PARAMETER	GATE	
■	091595.012	FL3-H	NO GATE	POS
—	091595.011	FL3-H	NO GATE	NEG



KEY	NAME	PARAMETER	GATE	
■	091595.014	FL3-H	NO GATE	POS
—	091595.013	FL3-H	NO GATE	NEG



KEY	NAME	PARAMETER	GATE	
■	091595.016	FL3-H	NO GATE	POS
—	091595.015	FL3-H	NO GATE	NEG

FIG. 18B

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

Intern. Application No.
PCT/US 97/06931

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category	Citation of document, with indication, where appropriate, of the relevant passages	
A	GIRI J G ET AL: "IL-15, A NOVEL T CELL GROWTH FACTOR THAT SHARES ACTIVITIES AND RECEPTOR COMPONENTS WITH IL-2" JOURNAL OF LEUKOCYTE BIOLOGY, vol. 57, no. 5, pages 763-766, XP000570466. ---	
A	WO 96 04306 A (SCHERING CORPORATION) 15 February 1996 see claim 20 see page 21 - page 22	1-3
A	WO 95 27722 A (IMMUNEX CORPORATION) 19 October 1995 see page 8, line 21 - line 38; claims	1
A	M.A. BLANAR ET AL: "interaction cloning : Identification of a helix-loop-helix zipper protein that interacts with c-Fos" SCIENCE, vol. 256, 15 May 1992, DC, pages 1014-1018, XP002042889 cited in the application see the whole document	16,17
P,X	29TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF NEPHROLOGY, 3 - 6 November 1996, NEW ORLEANS, LOUISIANA, USA, XP002042890 & D-W CHAE ET AL: "Mutant IL-15 protein exerting antagonistic effects on IL-15 triggered cell proliferation" JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, vol. 7, no. 9, November 1996, page 1654 see abstract A2029	1-11, 16-21
P,X	WO 96 26274-A (IMMUNEX CORPORATION) 29 August 1996 see claims; examples 1-4 see page 2, line 21 - page 4, line 4	1-3,5-7
P,X	D.K. PETTIT ET AL: "Structure-function studies of Interleukin 15 using site-specific mutagenesis, polyethylene glycol conjugation, and homology modeling" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 4, 24 January 1997, MD, US, pages 2312-2318, XP002042891 see the whole document	1-3,5-7
1		

INTERNATIONAL SEARCH REPORT

International application No

PCT/US 97/06931

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 22-34 and 35
because they relate to subject matter not required to be searched by this Authority, namely:
remark: Although these claims (as far as in vivo methods are concerned) are directed to a method of treatment of the human/animal body (rule 39.1(IV) PCT), the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest:

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/06931

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9604306 A	15-02-96	AU 3196195 A	04-03-96
-----	-----	AU 680909 B	14-08-97
WO 9527722 A	19-10-95	AU 6767394 A	30-10-95
		EP 0772624 A	14-05-97
		FI 963828 A	02-12-96
		NO 964141 A	06-12-96
-----	-----	-----	-----
WO 9626274 A	29-08-96	AU 5027596 A	11-09-96
-----	-----	-----	-----